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PROTEOLYSIS OF Z-DISC ACTIN

by

© PETER ANDREW NAGAINIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

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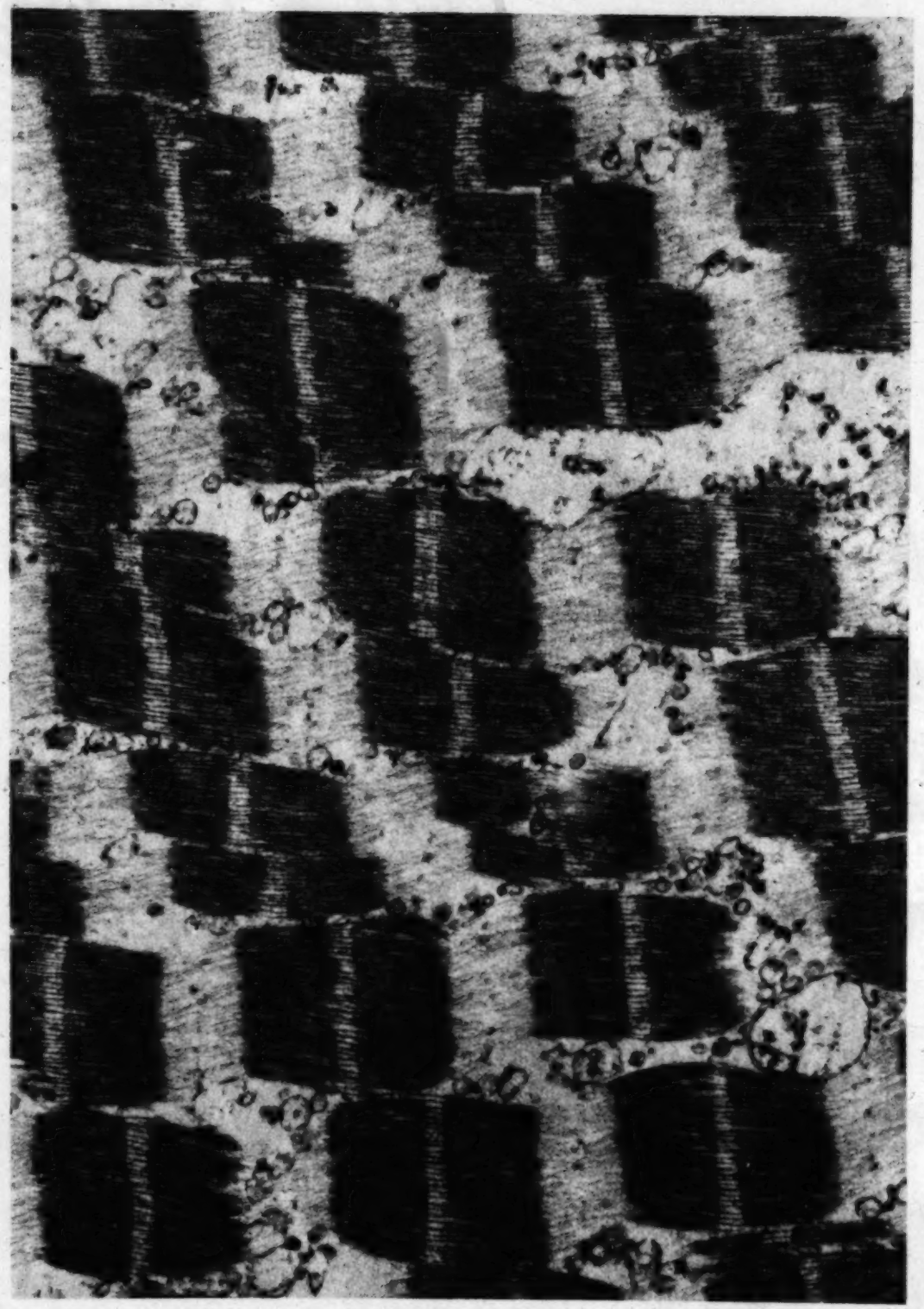

Z–discs removed by CAF

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled PROTEOLYSIS OF Z-DISC ACTIN, submitted by PETER ANDREW NAGAINIS in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Food Science.

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DEDICATION

To my Mom and Dad

ABSTRACT

Changes in the proteins of chicken myofibrils which occur during post-mortem aging were compared with the changes in isolated myofibrils incubated with purified calcium-activated factor (CAF). The effects of CAF were further examined by fractionating the myofibril proteins using Hasslebach-Schneider solution (H·S) and potassium iodide solution (KI). A small but constant proportion of myofibrillar actin was persistently insoluble in the KI extracted myofibril "ghosts" and was hydrolysable by CAF. The amount of actin in ghosts of CAF-treated or aged myofibrils was substantially reduced or completely eliminated.

The hydrolysis of ghost actin by CAF was studied and the reaction was found to have pH optimum and Ca^{2+} requirements similar to other CAF substrates.

Some properties of SDS-PAGE purified ghost actin were compared to those of KI soluble actin prepared by the same method. The amino acid composition was very similar but the isoelectric pH of the proteins differed.

The purified actins were used to immunize mice. Mice injected with KI soluble actin failed to produce antibodies in response to that protein. The antibodies produced against ghost actin recognized both actins identically. The fractionated antiserum used in indirect immunofluorescence experiments showed that the ghost actin resided in the Z-disc and was removed from that location by CAF. Thin filament actin was unaffected by CAF activity.

It was concluded that actin in the Z-disc may have a different conformation which could be responsible for its susceptibility to

hydrolysis by CAF. The proteolysis of Z-disc actin is likely the cause of Z-disc disruption observed due to aging or to incubation with CAF.

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TABLE OF CONTENTS

Chapter		Page
	DEDICATION	iv
	ABSTRACT	v
	ACKNOWLEDGEMENTS	vii
	LIST OF TABLES	xiv
	LIST OF FIGURES	xv
	LIST OF PHOTOGRAPHIC PLATES	xvi
1	INTRODUCTION	1
	GENERAL PHYSIOLOGY AND GROSS ANATOMY OF STRIATED MUSCLE	2
	THE MYOFIBRIL	3
	Muscle Contraction	5
	Myosin	6
	Actin	7
	The Thin Filament	7
	Contraction	9
	THE Z-DISC	11
	Ultrastructure	11
	Protein Composition	14
	Gap Filaments	16
	NON-MYOFIBRILLAR ACTIN	17
	Desmin Filament Actin	18
	THE CONVERSION OF MUSCLE TO MEAT	19
	Development of Rigor Mortis	20
	Tenderness Measurement	21
	Conditioning	22

Chapter		Page
1	Resolution of Rigor	23
	Proteolysis	24
	Alterations in Myofibrillar Proteins	25
	Calcium Activated Neutral Protease	26
	Statement of the Problem	31
2	MATERIALS AND METHODS	32
	PREPARATION OF PROTEIN SAMPLES	32
	Minced Muscle	32
	Myofibrils	32
	Isopycnic Centrifugation of Myofibrils	33
	Preparation of Myofibril Ghosts	33
	H·S and KI-Soluble Proteins	34
	Dialysis	34
	Aged Muscle	34
	Smooth Muscle Contractile Protein	35
	Actin Purification	35
	ASSAY OF PROTEOLYTIC ACTIVITY	36
	Assay of CAF Activity	36
	Hammarsten Casein	38
	Alkali-Denatured Casein	39
	Protein Concentration	39
	POLYACRYLAMIDE GEL ELECTROPHORESIS	39
	SDS-PAGE	39

Chapter		Page
2	Preparation of Gels	40
	Protein Sample Preparation	41
	Densitometry of Stained Gels	41
	Molecular Weight Determination	42
	Preparative SDS Gel Electrophoresis	42
	Final Purification of Proteins from Gel Slices	43
	Isoelectric Focusing	44
	AMINO ACID ANALYSIS	44
	IMMUNOLOGICAL STUDIES	45
	Preparation of Antisera	45
	Indirect Immunofluorescence	46
	ELECTRON MICROSCOPY	47
	Glycerinated Fibers	47
	Electron Microscopy of Glycerinated Fibers	47
3	RESULTS	49
	Chicken Breast Myofibrillar Protein	50
	Aged Chicken Myofibrils	55
	The Effects of CAF on Isolated Myofibrils	57
	Fractionation of CAF-Treated Myofibrils	61
	Extraction of Myofibrils From Aged Muscle	67

Chapter		Page
3	Hydrolysis of Extracted Myofibril Proteins by CAF	69
	Characteristics of KI Insoluble "Ghosts"	71
	Ca^{2+} Requirement for CAF Proteolysis	73
	pH Optimum of CAF Proteolysis	78
	Isolation of Pure Ghost Actin	78
	Hydrolysis of Isolated Actins by CAF	83
	CAF Hydrolysis of Denatured Myofibril Proteins	85
	Isoelectric Focusing of Isolated Actins	87
	CAF Hydrolysis of Smooth Muscle Protein	87
	Amino Acid Analysis of Isolated Actins	90
	CAF Proteolysis of Bovine Ghost Actin	92
	Production of Anti-Actin Antiserum	92
	Localization of Actin in the Z-Disc by Indirect Immuno-fluorescence	96
	ELECTRON MICROSCOPY	100
	Entire Muscle Fibers	100
	H·S Extracted Fibers	104
	KI Extracted Fibers	107
4	DISCUSSION	110

Chapter		Page
4	SUMMARY	126
	REFERENCES	127
	Appendices	
	A Purification of CAF from Chicken Breast Muscle	140
	B Extraction of CAF From Beef Muscle	146
	C Preequilibration of Dowex AG1-X2 Acetate Ion Exchange Resin	148

LIST OF TABLES

Table	Description	Page
1	Proteins of the Myofibril	53
2	Determination of Protein in Extracts of Control and CAF-Treated Myofibrils	62
3	Amino Acid Analysis of SDS-PAGE Purified KI Soluble and Ghost Actins	91

LIST OF FIGURES

Figure		Page
1.	The organization of skeletal muscle	4
2.	Regulation of muscle contraction by troponin	8
3.	Model of the vertebrate Z-disc	13
4.	Molecular weight determination by SDS-PAGE	52
5.	Effect of Ca^{2+} concentration on CAF activity	76
6.	Effect of pH on CAF activity using a KI insoluble ghost preparation as a substrate	79
7.	Effect of Ca^{2+} ion concentration on the activity of beef CAF on beef myofibrils	93
8.	Effect of pH on the activity of beef CAF on beef myofibrils	94
9.	Chromatography of the crude ammonium sulfate fraction containing CAF activity on DEAE-cellulose	142
10.	Elution profile of DEAE-purified CAF on Sepharose 6B	144
11.	Elution profile of chromatography of Sepharose purified CAF on a second DEAE column	145

LIST OF PHOTOGRAPHIC PLATES

Plate	Description	Page
1	Densitometry of SDS-PAGE of myofibrils from fresh and aged chicken pectoralis muscle	51
2	SDS-PAGE of myofibrils purified by sedimentation and by isopycnic centrifugation	56
3	Comparison of SDS-PAGE of changes in myofibril proteins from aged muscle with changes in myofibrils incubated with CAF	58
4	SDS-PAGE of myofibrils incubated for 14 hours with CAF prior to extraction with H·S and KI: Protein distribution in extracts	60
5	SDS-PAGE of myofibrils incubated one hour with CAF prior to extraction with H·S and KI: Protein distribution in extracts	66
6	SDS-PAGE of myofibrils prepared from fresh and aged muscle, then extracted with H·S and KI: Protein distribution in extracts	68
7	SDS-PAGE of H·S and KI protein extracts and insoluble residues after incubation with CAF	70
8	SDS-PAGE of purified G-actin and F-actin incubated with CAF: Comparison of actin hydrolysis in KI insoluble ghosts and isolated actin samples ...	72
9	SDS-PAGE of KI insoluble ghost proteins extractable in SDS with and without 2-mercaptoethanol ...	74
10	SDS-PAGE of myofibril ghosts incubated with CAF at increasing Ca^{2+} ion concentration	77
11	SDS-PAGE of myofibril ghosts following incubation with CAF at increasing pH values	80
12	SDS-PAGE of KI soluble and ghost actin isolated by preparative gel electrophoresis	82
13	SDS-PAGE of myofibrils, ghosts, and isolated actins: Time course of hydrolysis by CAF	84
14	SDS-PAGE of myofibrils and KI soluble myofibril protein denatured with NaOH and incubated with CAF	86

Plate	Description	Page
15	Isoelectric focusing of actins isolated by preparative gel electrophoresis	88
16	SDS-PAGE of smooth muscle protein incubated with CAF	89
17	SDS-PAGE of beef myofibrils, H·S and KI extracted myofibrils and isolated actin incubated with beef CAF	95
18	Double-immunodiffusion of fractionated anti-ghost actin serum against isolated KI soluble and ghost actin	97
19	Indirect immunofluorescent labelling of ghost actin in myofibrils, KI extracted myofibrils and myofibrils incubated with CAF	99
20	Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM EDTA	101
21	Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM $CaCl_2$	103
22	Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM EDTA and subsequently extracted with H·S	105
23	Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM $CaCl_2$ and subsequently extracted with H·S	106
24	Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM EDTA and subsequently extracted with H·S and KI	108
25	Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM $CaCl_2$ and subsequently extracted with H·S and KI	109

Chapter 1

INTRODUCTION

This study is directed toward the composition and structure of the Z-disc and its breakdown in striated muscle myofibrils. The Z-disc serves as the anchor point for the contractile elements and confers strength and continuity to muscle myofibrils in living tissue. Degradation of the Z-disc structure has been implicated in the weakening of living muscle tissue in muscular and neuromuscular disorders. In post-mortem muscle, Z-disc degeneration is thought to contribute to the tenderization of muscle processed for consumption as meat.

Recently the Z-disc has been the subject of considerable research, which revealed some of its protein components, its ultrastructure, and its enzymatic breakdown by endogenous muscle enzymes. However, the actual mechanism of Z-disc dissolution has not yet been made clear.

In this study, evidence is presented that may explain how endogenous enzymes break down the Z-disc structure.

In order to introduce the contribution of the Z-disc degradation to the development of tenderness in post-mortem muscle, a brief description of overall physiology of muscle is included. The structure and interaction of the proteins of the contractile system and the mechanism of muscle contraction are presented in order to introduce the events surrounding the development of rigor mortis in post-mortem muscle. The subsequent factors that may contribute to the development of tenderness in meat are also discussed.

GENERAL PHYSIOLOGY AND GROSS ANATOMY OF STRIATED MUSCLE

Striated muscle is a highly organized system of tissues that permits voluntary movement in higher organisms. This system is composed of organized contractile and structural elements in association with connective tissue, a vascular system, and a nervous network.

Connective tissue gives support and strength to the muscle structure as a whole, and also serves as a link in the transmission of the physical force generated between the muscle fibers and the skeletal system. Muscle connective tissue is classified into three categories. The epimysium, a connective tissue sheath, surrounds the entire muscle and is continuous with both the perimysium, which penetrates the muscle and divides its structure into bundles of fibers, and the endomysium which surrounds each fiber. The connective tissue is primarily composed of collagen but also contains some elastin and reticulin.

Throughout the muscle structure, a fine network of blood vessels and nerves can be found. The blood vessels provide necessary nutrients and oxygen required for energy metabolism, and remove the byproducts and heat generated in contraction. The nerves provide the signal for unified contraction as will be described in a subsequent section.

The individual contractile unit of muscle is the muscle fiber, a multinucleated cell that may extend for several centimeters in the muscle. Muscle fibers make up seventy-five to ninety-two percent of the total muscle volume, the remainder being connective tissue, blood

vessels, nerve fibers and extra-cellular fluid (Forrest, Aberle, Hedrick, Judge, and Merkel, 1975) (Figure 1).

Surrounding each muscle fiber is an excitable membrane system, the sarcolemma, which is the key to the control of muscle contraction. The sarcolemma and the connected network of T-tubules transmit the contractile stimulus from the nervous system to each myofibril in the fiber virtually simultaneously.

Contained within each fiber may be as many as 2,000 or more myofibrils, bathed in the sarcoplasm (or cytosol) which contains the enzymes and accessory compounds required for muscle contraction.

Individual fibers when viewed in the light microscope appear striated having a regularly repeating pattern of light and dark bands. The characteristic light isotropic (I) zone is bisected by the Z-line (more accurately the Z-disc due to the cylindrical nature of the sarcomere in cross-section) and the dense anisotropic (A) zone bisected by the H-zone and the M line are easily recognizable features of muscle ultrastructure. Because adjacent myofibrils are joined and the bands appear in register, the resulting uniformly banded appearance characteristic of skeletal muscle led it to be also known as striated muscle.

THE MYOFIBRIL

This section briefly outlines the muscle proteins and the mechanisms of muscle contraction, and is not designed as a comprehensive review, but merely serves to introduce the structure and function of the proteins of the myofibril.

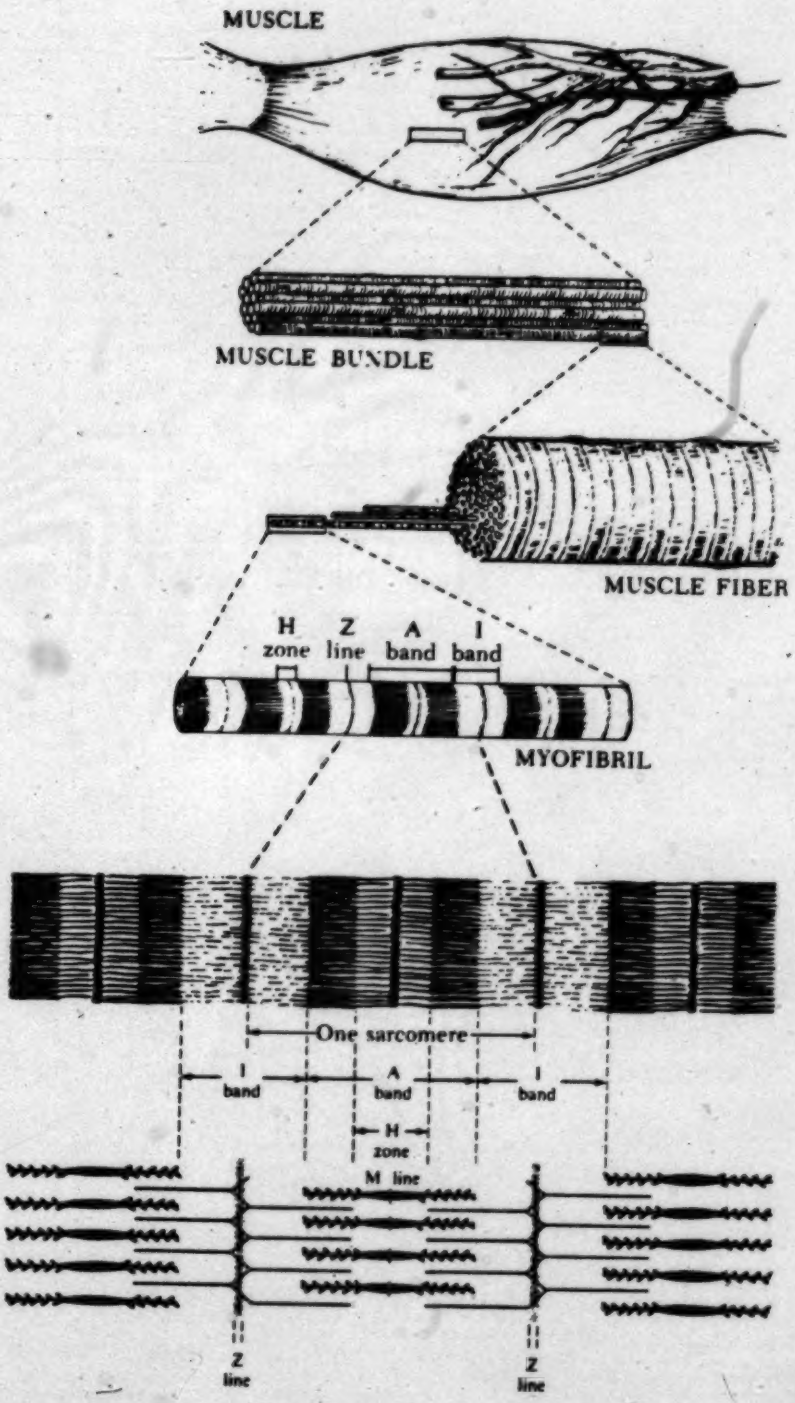

Figure 1. The organization of skeletal muscle.

The myofibril is a highly organized matrix of water insoluble proteins, some of which generate the mechanical force of contraction, and others which provide the structural foundation to which the contractile elements are anchored. While the major contractile and regulatory proteins of the myofibril have been identified, the structure and function of many of the minor components remain uncertain.

The introduction of the sliding filament theory of muscle contraction (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954), was based on observations of the behaviour of the myofibril striations during contraction. Shortly thereafter, in 1957, H.E. Huxley presented electron microscope evidence of muscle sections which showed cross-bridge connections linking thick and thin filaments, thus providing the basis for the cross-bridge mechanism and the generation of force through the consumption of ATP. These vital observations became the basis of our current understanding of muscle contraction, namely the "sliding filament theory."

Muscle Contraction

It is now accepted that the contraction of muscle involves the coordinated action of contractile and regulatory proteins. The force of contraction is generated by the sliding of the thick and thin filaments past one another in unison. The thick filament is composed entirely of myosin, a highly asymmetric molecule composed of two identical subunits or heavy chains. Each has a globular head joined to a long α-helical polypeptide tail portion by a trypsin sensitive "hinge" portion. Associated with the head region, there exist two identical and two non-identical light chains. The entire

myosin complex is thus composed of six polypeptide chains with a total molecular weight of approximately 460,000 daltons. The myosin head region has been shown to possess ATPase activity and can also bind thin filament actin. These last two key functions are fundamental to the contractile mechanism.

Myosin

Myosin molecules are arranged in the thick filament in a regular helical packing arrangement so that the myosin heads project from the filament at regular intervals of 143Å (Huxley and Brown, 1967). In each filament, the myosin molecules are arranged in such a way that the heads project outward from the centre axis of the filament and are staggered at 120° to each other. Assembly is accomplished by tail to tail aggregation of the myosin molecules in such a way that the heads point away from the centre of the molecule. The centre portion of each thick filament, a bare region where reversal of polarity of the molecules occurs and where no myosin heads protrude, causes the pseudo-H-zone at the centre of the sarcomere.

The myosin tail regions are probably bound together by C-protein, which is thought to surround the thick filament at regular intervals (Offer, 1972; Offer, Moos, and Starr, 1973). Another set of proteins are the M-proteins which may also ring the thick filaments at the M-line. The C- and M-proteins possibly serve the function of organizing the myosin in the thick filament, contribute to its structural integrity and help maintain the filaments in register.

Actin

The thin filament is composed primarily of actin and the regulatory proteins troponin and tropomyosin. The globular (G) actin monomer is a roughly spherical protein of 41,780 daltons containing 374 residues as determined by sequence data (Elzinga, Collins, Kuehl, and Adelstein, 1973) but may exhibit varying molecular weights from 43,000 to 50,000 daltons when measured by ultracentrifugation (Rees and Young, 1967) and by SDS polyacrylamide gel electrophoresis. Actin contains the unusual amino acid N^T-methyl histidine and also has an acetylated N- terminal residue. It contains five cysteine residues but no disulfide bridges. A high proline content results in low α-helical content of only fifteen to twenty percent.

Actin, an ubiquitous molecule, is found not only in striated and smooth muscles but also in non-muscle cells (Pollard and Weihing, 1974). It has been implicated in many of the cell functions where physical movement occurs.

The Thin Filament

G-actin monomers polymerize to form a double-stranded helix in the presence of ATP. In the process of polymerization, each G-actin monomer binds one mol of ATP/mol monomer (Rees and Young, 1967; Tsubori, 1968). The ATP is split into ADP and inorganic phosphate during the polymerization of G-actin to F-actin filaments. Little is actually known about the role of ATP hydrolysis in polymerization or about the binding sites between actin molecules. Electron micrographs of reconstituted filaments indicate a double helix of monomers in register with a width of 60Å and a half pitch of 365Å (Huxley, 1963). In the "grooves" formed between the two actin

strands lie the regulatory proteins, tropomyosin and troponin. Tropomyosin, a long, two-chained twisted coil composed of two non-identical α-helical subunits, lies end to end in the groove, spanning seven actin monomers. The tropomyosin molecules are arranged in such a way that each molecule does not lie exactly in the bottom of each groove but slightly to one side so that it only makes contact with one actin chain, as illustrated in Figure 2.

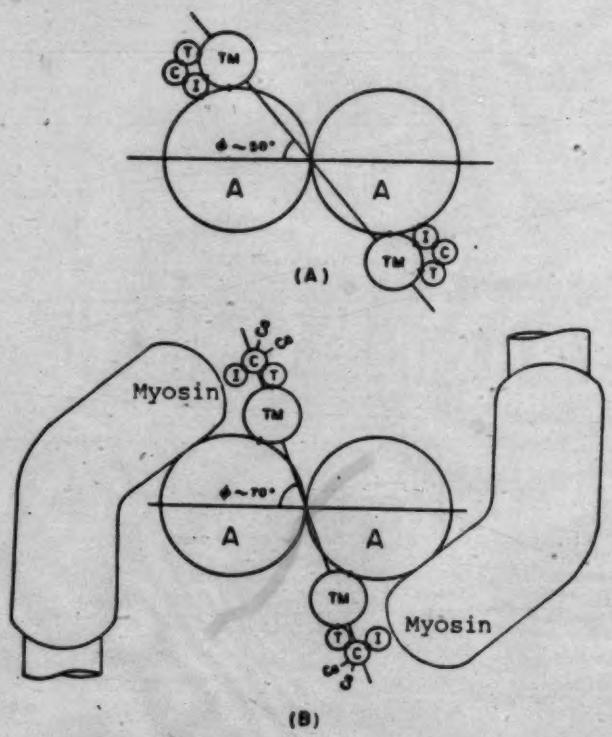

Figure 2. Regulation of muscle contraction by troponin. A. Relaxation in the absence of Ca^{2+}. B. Activation. A, actin; TM, tropomyosin; T, TN-T; I, TN-I; and C, TN-C. Interactions between proteins are indicated by a short connecting line (from Potter, J.D., and Gergely, J., Biochemistry, 1974, 13, 2702).

Associated with each tropomyosin molecule exists a troponin complex. This globular protein is composed of three different subunits each of which has its own unique function. Troponin-C (TN-C) has a site for the binding of four molecules of calcium per molecule

of TN-C. The inhibitory subunit TN-I functions to block the interaction of the myosin head with actin. TN-T, the tropomyosin binding subunit, permanently links the other subunits to tropomyosin. Troponin can also bind a site on actin depending on the availability of Ca^{2+} as will be discussed shortly.

The protein β-actinin has also been shown to be associated with the thin filament although it is apparently not required for contraction. Its precise location has not yet been established.

Contraction

Contraction in striated muscle is triggered by a nerve impulse. A wave of depolarization travelling down a nerve fiber bridges the neuromuscular junction by causing the secretion of the neurotransmitter acetylcholine at the motor-end plate. The acetylcholine triggers the depolarization of the muscle cell sarcolemma which rapidly spreads over the entire surface of the muscle filament, and at the same time is conducted inward through the fiber by the T-system, a network of tubules surrounding every myofibril near the Z-disc. This intricate system transmits the depolarization signal that initiates muscle contraction in every myofibril virtually simultaneously.

Adjacent to the T-tubules at the Z-disc are the terminal cisternae, a separate membrane system to which the depolarization signal is passed. The terminal cisterna forms an elaborate collar-like ring around each Z-disc and also surrounds the length of the myofibril with a perforated membrane sheath. This system of vesicles known collectively as the sarcoplasmic reticulum (SR) is responsible

for the conversion of the action potential impulse to the chemical impulse which triggers contraction.

In the relaxed state of muscle, the SR regulates the equilibrium between free and SR-sequestered calcium in the sarcoplasm bathing the myofibril. This is accomplished by active transport of Ca^{2+} ions at the expense of ATP across the membrane into the SR system. In resting muscle, the free Ca^{2+} concentration is normally less than 10^{-7} M. Within the SR, Ca^{2+} may be sequestered by two or more Ca^{2+}-binding proteins. In some muscles, the mitochondria may also cooperate with the SR in sequestering Ca^{2+}.

The depolarization of the SR membrane system interferes with the equilibrium distribution of Ca^{2+} by momentarily interfering with the active transport system. The subsequent efflux of Ca^{2+} increases the sarcoplasmic Ca^{2+} concentration one hundred fold and initiates contraction. TN-C on the thin filament binds this Ca^{2+} and undergoes a conformational change which somehow is transmitted to the entire troponin-tropomyosin complex. TN-I becomes unbound from actin and the entire complex rolls tighter into the groove in the thin filament, thus exposing the myosin binding site on actin. The myosin head then binds the actin molecule. Each bound myosin head is believed to undergo a conformational change as it releases the stored energy from the hydrolysis of a molecule of ATP. The change (via a mechanism which still remains unexplained) results in a change in the orientation of the myosin head. This shift in attitude (often called the power stroke) is believed to be the step in which the chemical energy of ATP is converted to motion by an alteration in the spatial relation-

ship between the actin and myosin molecules. In this way, by repeated attachment and detachment of the myosin heads, the thick filament moves itself along the thin filament. Thus the tension developed in muscle is in direct proportion to the number of cross bridges formed between the thick and thin filaments.

Relaxation of the muscle occurs when the neural impulse is terminated. The membrane systems rapidly become repolarized, Ca^{2+} again is sequestered in the SR. When the sarcoplasmic Ca^{2+} levels drop to the resting level the actin-myosin interaction once more becomes inhibited, the thick and thin filaments become detached from one another and can slip freely past each other to a rest position.

During relaxation the force for the return of the filaments to the normal resting position is provided by the tension stored during contraction, and by the elastic connective tissue net which surrounds the fibers.

It is important to note here that ATP is required for relaxation to occur. Each myosin head must bind a molecule of ATP in order to become detached from the adjacent actin strand. In the absence of ATP, the actin and myosin become irreversibly locked in a rigor bond.

THE Z-DISC

Ultrastructure

The early observations on muscle contraction showed that as the muscle filaments slide past one another, the Z-discs are drawn closer together. As more knowledge was gained about the structure of

the myofibril and the role of the thick and thin filaments in muscle contraction it became apparent that the Z-discs have structural importance in anchoring and organizing the thin filaments in space. Until fairly recently the structure and composition of the Z-disc had remained relatively unknown.

Much of the current knowledge pertaining to the Z-disc has been developed from observations of its physical appearance in electron micrographs. One of the first published studies on the vertebrate Z-disc (Knappeis and Carlson, 1962) established that the I filaments on either side of the Z-disc were out of register with each other. They also noted that rod-like filaments (Z-filaments) project from the ends of the I filament. As the Z-filaments angled and coursed through the Z-disc, they formed a tetragonal pattern when viewed in cross-section. In the original model it was proposed that each thin filament on one side of the Z-disc was connected to the four filaments opposite it through the Z-disc by means of the Z-filaments. The resulting model produces a basket-weave pattern, tilted 45^{o} from the orientation of the thin filaments, that closely resembles the pattern commonly seen in sections of the Z-disc (Ullrick, Toselli, Saide, and Phear, 1977).

However, different interpretations of the basket-weave pattern have led to widely differing models of Z-disc structure. Because the size of both the thin filaments (60Å) and the proposed Z-filaments approaches the limit of resolution of the electron microscope, the results obtained from examination of micrographs can be interpreted in many ways. As well, in some samples artifacts were introduced during fixation of the EM samples that superimposed "large" and "small" lattice patterns upon the basic basket-weave (Landon, 1970; MacDonald and Engel, 1971).

As a result of the above problems several different models were proposed.

The model which appears to fit most of the evidence was developed by Ullrick et al. (1977), and is presented in Figure 3.

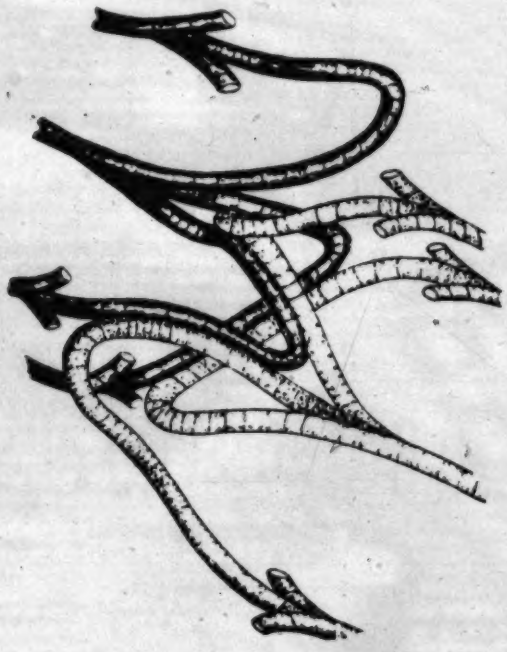

Figure 3. Model of the vertebrate Z-disc.

Their model involved the splitting of thin filaments into two or three strands which loop into the Z-disc and re-emerge to join other filaments on the original side. Some amorphous material may exist at the centre of the Z-disc. This model is supported by evidence that the Z-disc has been observed to split into two halves (Maron, Ferrans and Roberts, 1975; Sjostrand, 1962) and therefore thin filaments located on opposite sides of the disc are probably not contiguous.

Protein Composition

Huxley and Hanson (1954) determined that the Z-disc accounts for less than ten percent of all the myofibril protein. Until very recently, the only protein that had been identified in the Z-disc was α-actinin. Fluorescent antibody techniques (Masaki, Endo, and Ebashi, 1967; Scholl-meyer, Goll, Robson, and Stromer, 1973) and selective extraction studies (Briskey and Fukazawa, 1970; Etlinger and Fishman, 1972; Fukazawa and Bris-kay, 1970a, b; Goll, Mommaerts, Reedy, and Seraydarian, 1969; Robson, Goll, Arakawa, and Stromer, 1970) revealed conclusively that α-actinin resides in the Z-disc.

Early reports of its biochemical activity (Ebashi and Ebashi, 1965; Maruyama and Ebashi, 1965; Seraydarian, Briskey and Mommaerts, 1967; Temple and Goll, 1970) established that α-actinin interacts with actin to form an insoluble gel complex. α-Actinin was also found to affect rates of actomyosin superprecipitation and actomyosin ATPase activity (Ebashi and Ebashi, 1965) but has been shown to be unneces-sary for muscle contraction (Briskey, Seraydarian, and Mommaerts, 1967; Seraydarian et al., 1967). Using purified α-actinin, Goll, Suzuki, Temple, and Holmes (1972) were able to show that at physio-logical temperatures (37°C), α-actinin binds only one end of the thin filament, the Z-disc end, and that this binding could be inhibited by tropomyosin. Assuming a molecular weight of 200,000 daltons for α-actinin it was calculated that one molecule of α-actinin may bind ten to eleven molecules of actin. This ratio corresponds to one molecule of α-actinin per turn of the F-actin helix (Goll et al., 1972).

Using fluorescent antibodies, Granger and Lazarides (1978) demonstrated that actin also exists in the interior of the Z-disc.

Within the Z-disc α-actinin and actin probably interact, forming the insoluble complexes that have been described above.

Actin also exists at the periphery of the Z-disc where it may be involved in the structure of filaments that contain the newly identified proteins desmin (Granger and Lazarides, 1978; Lazarides and Balzer, 1978) and vimentin (Granger and Lazarides, 1979).

Desmin (50,000 daltons) and vimentin (52,000 daltons) both are recently discovered muscle proteins. In association with actin, these proteins form a distinct class of filaments in both smooth and striated muscle and also play important roles in cellular structure and movement. These filaments have become known collectively as the intermediate (or 100 Å) filaments, so named because their size falls between that of the thin (actin) filaments (60 Å) and the thick (myosin) filaments (150 Å) and the microtubules (250 Å).

Little has been determined about the structure and properties of desmin and vimentin. Both molecules are insoluble at high salt concentrations, but are solubilized at low pH or by agents that dissociate hydrophobic bonds such as SDS. Isolated desmin co-polymerizes with actin to form 100 Å filaments (Hubbard and Lazarides, 1979). Together, all three form an elastic network of intermediate filaments that connect adjacent Z-discs.

The Z-disc can then be seen as an organizational center of the myofibril structure. The lattice of thin filaments is anchored in the Z-disc via links with α-actinin. Adjacent Z-discs are linked to each other and maintained in register by the intermediate filaments. The intermediate filaments may also form the link between the Z-disc network

and the membranous organelles such as the T-system and the sarcolemma (Lazarides, 1980).

Gap Filaments

Several authors have reported the existence of a third type of filament in myofibrils. Evidence for these structures comes primarily from studies of electron micrographs. Guba, Harsanyi, and Vajda (1968a) reported filaments connecting the Z-discs of myofibrils where myosin and much of the actin had been extracted. These filaments could no longer be observed after extraction with KI. Guba et al. (1968b) reported that the hypothetical protein fibrillin could be prepared from the KI extracts, and suggested that these fibers were composed of that protein. Based on cross-sections of extracted fibers, Guba and his associates proposed a continuous filament system forming the core of the thick filaments and passing through the Z-disc to connect the thick filaments of adjoining sarcomeres. However, little popular support has been profferred for these suggestions.

Somewhat later, in a series of electron microscope observations on single overstretched fibers, Locker and Leet (1975, 1976a, 1976b, 1976c) and Locker, Daines, and Leet (1976) observed filaments similar in appearance and location, but which resisted extraction in M KI. Locker concluded that a series of elastic filaments 80 Å in diameter ran continuously through the Z-discs and the thick filaments.

Several other authors also reported fibers of similar (dos Remedios, 1978) and of differing diameters (Maruyama, Matsubara, Natari, Nonomura, Kimura, Ohashi, Murakomi, Handa and Eguchi, 1977; Walcott and Ridgeway, 1967). Although little biochemical evidence exists to support the

existence of gap filaments, it is difficult to explain the observation that some material remains within the myofibril which prevents the Z-discs from separating following extraction of myosin and actin (dos Remedios and Gilmour, 1978; Granger and Lazarides, 1978; Huxley and Hanson, 1957).

NON-MYOFIBRILLAR ACTIN

It is becoming increasingly evident that actin is involved in numerous other cell functions. In non-muscle cells, actin and myosin have been implicated in a number of contractile and structural functions including cytokinesis, exocytosis, endocytosis, cell adhesion to a substratum, cell locomotion, membrane ruffling and maintenance of cell shape (as reviewed by Pollard and Weihing, 1974; Lazarides, 1976, 1980).

Actin in most of these cases has been found to exist in thin (60 Å) filaments. Using antibodies to actin, Lazarides (1976) demonstrated an extensive three-dimensional network of actin filaments in cultured cells. The organization of these filaments undergoes constant metamorphosis. The actin filaments continuously assembled and disassembled in areas of the cell where movement occurs (Lazarides, 1976). These actin filaments appear to have similar protein constitution to the thin filaments of striated muscle. Tropomyosin is sometimes found associated with the actin along the length of the filament and may stabilize the actin filament. Structures containing α-actinin (analogous to Z-discs) exist at vertices where several actin filaments are joined.

Movement in non-muscle cells may therefore take place in a similar manner to striated muscle, through the cooperation of actin and myosin, and regulated by tropomyosin.

Desmin Filament Actin

Recently a new class of intermediate (100 Å) filaments has been identified and named the desmin filaments. These filaments have been shown to consist of non-stoichimetric amounts of desmin and actin (Hubbard and Lazarides, 1979). These filaments form the highly insoluble network linking adjacent Z-discs in striated muscle described previously. While actin in the desmin filaments may be immunologically identical with thin filament actin Lazarides speculated that this actin is likely to be in a different conformational form.

Actin has been observed to be a highly conserved molecule in nature. Striated muscle actin from avian and mammalian sources is generally accepted as indistinguishable by the criteria of peptide mapping and amino acid composition (Carsten and Katz, 1964). This may account for the fact that native actin from one species will not elicit the production of antibodies in many different species.

However, subtle heterogeneity does exist even in actins extracted from a single animal. Actin from chick brain cells in comparison to chicken skeletal muscle actin exhibited no difference in biochemical properties (apparent molecular weight, polymerization-depolymerization properties, activation of myosin ATPase and decoration by heavy meromyosin) (Pollard and Weihing, 1974). However, slight differences were detected on the basis of tryptic peptide maps (Gruenstein and Rich, 1975), in the sequences of selected peptides (Elzinga, Maron, and Adelstein, 1975), and in SDS-urea polyacrylamide gel electrophoresis (Storti, Coen, and Rich, 1976; Storti and Rich, 1976). Whalen, Butler-Browne, and Gros (1976) used two dimensional isoelectric-focusing (IEF) to resolve actin into three distinctly different isoelectric variants, identified as α,

β, and γ-actin on the basis of their electrophoretic mobility. α-actin appears to be the predominant but not exclusive form in skeletal muscle, and the γ-form prevails in smooth muscle, developing muscle and non-muscle cells.

It is probable that these observed actin variants arise from different genes rather than post-translational protein modification (Gruenstein and Rich, 1975; Izant and Lazarides, 1977). Therefore, while all actins were at first thought to be identical, a considerable amount of evidence now indicates that minor heterogeneity exists that may influence the role of actin within the cell.

THE CONVERSION OF MUSCLE TO MEAT

Muscle is a superbly efficient biochemical machine, splendidly organized to convert biochemical energy into strength and motion. It is, however, also very good to eat.

The fact that muscle contains all essential amino acids and to a lesser extent, essential vitamins and minerals, makes it an excellent source of human nutrition. It has been observed for perhaps hundreds of years that changes occur in post-mortem muscle that alter the properties of meat. Immediately following death and exsanguination of the animal a characteristic shortening of the muscle or isometric tension development occurs and leads finally to inextensibility of the muscle. These changes are known collectively as the onset of rigor mortis. The study of the biochemistry of living muscle has now allowed us to explain these post-mortem events. The explanation of the molecular metabolism during contraction was crucial to our comprehension of the

factors that contribute to the development of rigor mortis and ultimately affect the quality of meat.

Development of Rigor Mortis

In the living animal, the circulatory system performs a number of functions essential for the maintenance of homeostasis within the muscle. The circulatory system provides the muscle system with the essential nutrients and oxygen required for energy metabolism. It also removes the byproducts and heat generated during energy metabolism. Death of the animal by exsanguination results in a complex series of changes that occur within the muscle tissue.

Circulatory failure deprives the muscle of 0_2, the terminal electron receptor, required for the oxidative phosphorylation necessary to maintain a constant level of ATP essential for myofibril contraction. In an effort to replenish the ATP supply, the muscle metabolism shunts available glucose into the less efficient glycolytic pathway.

Existing ATP is rapidly catabolized by the muscle's endogenous actomyosin and sarcoplasmic reticulum ATPases. The accumulation of lactic acid, a byproduct of the rapidly glycolysing metabolism results in a characteristic pH fall from pH 7.2 in normally metabolizing muscle, to an ultimate pH between 5.4 and 6.2. The rapidly dwindling ATP supply and the drop in pH both contribute to the gradual loss in the ability of the SR and the mitochondria to sequester Ca^{2+}. As a result, the newly freed Ca^{2+} slowly leaks into the sarcoplasm and elicits a slow, weak muscular contraction similar in many aspects to the normal contraction of muscle (Davies, 1966). This contraction contributes even more to the increased ATP deficit, and continues until, eventually, the ATP drops to a suffic-

iently low level (below 0.1 μ moles/g muscle) that contraction ceases (Bendall, 1973). The actin and myosin molecules in the absence of ATP are irreversibly linked in so-called "rigor" bonds. As a result, the normally extensible muscle becomes stiff and resists stretching (Davies, 1963).

The rate of rigor development varies greatly among species, among animals within a species, and even varies among different muscles in the same animal. Typically, in beef the maximum tension development may occur between twelve and up to twenty-four hours post-mortem (Cia and Marsh, 1976; Currie and Wolfe, 1980; Dransfield and Rhodes, 1975; Nuss and Wolfe, 1981) but may develop in chicken in as quickly as three to six hours (Wood, 1973).

Maximum tension development signals the end of the primary events of rigor mortis. At this time, organoleptic toughness is maximal, but with subsequent conditioning, tenderness has been demonstrated to increase. Increased fragility results in decreased rigidity and an increase in perceived tenderness.

Tenderness Measurement

The mechanical measurement of the parameters of meat texture and tenderness would seem simple at first to the casual observer. However, after the development of countless methods, the solution is still far from perfect, and a suitable universal method has not been agreed upon.

One of the first devices developed for the purpose of measuring meat tenderness (Lehmann, 1907) involved two mechanical methods, one to record breaking strength of muscle, and the other measured the force

required to bite through the sample with two cutting edges. Although many other similar devices have been reported, the Warner-Bratzler shear cell introduced in 1928 appeared to best parallel results of taste panel studies. The device measures the force required to shear a round plug of cooked meat. Although wide variations in results can be introduced by equipment and methods used in sampling and cooking, results of studies using this device for mechanical texture measurement, often in conjunction with studies using trained taste panels, provide the basis for value judgements of meat tenderness.

Conditioning

For centuries, it has been common practice to allow meat to age from a few days to several weeks or months in some instances to improve the quality. As early as 1874 (Bouley, 1874), conditioning was thought to be responsible for the development of a significant increase in tenderness and flavour. Similarly, in 1907, Lehman described the first mechanical measurement of the improvement of tenderness with conditioning. Since that time, it has become established fact that muscle undergoes a process of tenderization following the development of rigor mortis (Bate-Smith, 1948; Bendall, 1973).

Several explanations of the tenderization observed during conditioning have been proposed. The reversal or breaking of some of the rigor bonds formed between the thick and thin filaments might allow the muscle to relax and become extensible once more (Wierbicki, Kunkle, Cahill, and Deatherage, 1954). Other explanations involve the hydrolysis of certain structural components within the myofibril that may account for increased fragility of the structure (Dayton et al., 1975),

yet another hypothesis involves the alteration of the state of the water with pH surrounding the protein filaments of the myofibril (Currie and Wolfe, 1980; Hamm, 1960). In many of the experiments designed to test these explanations, it was impossible to distinguish which observations were due to changes in the muscle, and which were merely effects of other post-rigor changes. In addition, it may be possible that all the proposed mechanisms contribute to some extent to the final tenderization, hence it is difficult to distinguish the contribution of each process to final improvement in quality.

The following is a summary describing results of current research into the most likely mechanisms of post-rigor tenderization.

Resolution of Rigor

Because the formation of rigor bonds was hypothesized to be the cause of the rigid state of muscle in rigor, the reversal or so-called "resolution" of rigor mortis was thought likely to involve the dissociation of the actomyosin complex into actin and myosin by breaking of the "rigor bonds." While early workers argued that total dissociation did occur (Partmann, 1963; Takahashi, Mori, Nakamura, and Tonomura, 1965; Takahashi, Fukazawa, and Yasui, 1967), and that sufficient ATP remained in the muscle to partially dissociate or weaken the rigor complex (Fujimaki et al., 1965; Goll, 1968), it has now generally held that rigor bond formation is an irreversible process (Davies, 1963). These views are substantiated by evidence gathered from several different lines of research. Gothard, Mullins, Boulware, and Hansard (1966) have shown that sarcomere length decreases pre-rigor but that no change in the sarcomere length can be observed in post-rigor muscle samples, indicating

that the thick and thin filaments remain firmly linked.

Other experiments using purified actin and myosin could distinguish no differences in the ATPase activity (Hay, Currie, and Wolfe, 1972, 1973) or changes in the viscosity of the actomyosin complex (Jones, 1972; Samejima and Wolfe, 1976) that would indicate a change in the state of the actomyosin complex during conditioning.

Proteolysis

The earliest observations of changes occurring in conditioned muscle coincided with development of advanced equipment and techniques for the electron microscope (EM) during the 1960s. It was then observed that with increasing periods of conditioning, progressive disruption of myofibrils occurred (Davey and Gilbert, 1967). Alterations of the observed Z-disc structure were the most prominent. A marked decrease in the density of the Z-disc was reported (Fukazawa and Briskey, 1970a; Fukazawa and Yasui, 1967; Stromer and Goll, 1967). In chicken muscle, these changes can be observed within twenty-four hours at $2^{o}C$ (Hay et al., 1973), while in beef or pork similar changes may occur only after a week (Olson, Parrish, Dayton and Goll, 1977).

The weakening of the myofibril coincides with the degradation of the Z-disc; breaks are frequently observed in that region. EM study has revealed that in post-rigor muscle, the myofibril becomes susceptible to fracture at the Z-disc or at the I-Z junction when stretched (Davey and Dickson, 1970; Gann and Merkel, 1978). This observation has been used in the evaluation of the fragility of post-rigor myofibrils. Homogenized muscle breaks into shorter myofibril segments, with increased periods of conditioning (Fukazawa, Briskey, Takahashi, and Yasui, 1969).

These observations were later used to relate conditioning to subjective tenderness (Moller, Vestergaard, and Wismer-Pederson, 1973).

Not all muscles are affected by conditioning to the same extent. Dutson, Pearson and Merkel (1974) and Hay et al. (1973) demonstrated that the fiber type of individual muscle determined to what extent the Z-discs were affected. Both studies agreed that damage to Z-discs was extensive in muscles of high white fiber content, while predominantly red muscles showed little or no change in structure.

Alterations in Myofibrillar Proteins

Observations on the increased fragility of the myofibrils in conditioned muscle, and that breaks occurred at the Z-disc, suggested that changes may occur in the proteins of the myofibril. The refinement of techniques for protein separation and analysis, particularly polyacrylamide gel electrophoresis (PAGE), have greatly facilitated the advancement of our knowledge of myofibrillar proteins and their degradation.

As early as 1917, Hoagland, McBride and Powick, had measured an increase in "non-coagulable nitrogen" with storage time. That study and several others that followed indicated the activity of endogenous proteolytic enzymes. However, little specific information about the kinds and functions of muscle proteases was available at that time.

Cathepsins were one of the earliest classes of proteases identified in muscle cells. Cathepsins are stable proteolytic enzymes with low pH optima which are generally found in the lysosomal fraction of cell homogenates. They are responsible for the degradation of proteins which have been enveloped by lysosomes. In muscle, protein degradation

was at first attributed to the cathepsins after their identification in the muscle (as reviewed by Bate-Smith, 1948). It has since been established that muscle tissue does contain several classes of lysosomal cathepsins. Cathepsin D is the most abundant of these enzymes (Canonico and Bird, 1970). Traces of cathepsin B (Schwartz and Bird, 1977), cathepsin A (Iodice, Leong, and Weinstock, 1966) and recently cathepsin L (Okitani, Matsukura, Kato, and Fujimaki, 1980) have also been detected. It has been established that cathepsins B, D and L have the ability to degrade actin and myosin, and that L can also degrade α-actinin under optimum conditions (Okitani et al., 1980; Schwartz and Bird, 1977). Penny (1980) argued that although these cathepsins may be active at the ultimate pH range of muscle (pH 5.5-6.2), no such hydrolysis of these proteins is observed in aging experiments (Eino and Stanley, 1973a, 1973b; Okitani et al., 1972; Penny and Ferguson-Pryce, 1979; Samejima and Wolfe, 1976). It therefore seems likely that cathepsin activity does not contribute greatly to the tenderization of muscle during conditioning. Furthermore, Eino and Stanley (1973b) contend that the primary substrate of the cathepsins is the sarcoplasmic protein.

Calcium Activated Neutral Protease

The importance of a calcium activated neutral protease (CANP) in the conditioning of muscle had been overlooked completely until its discovery in the early 1970s. The enzyme, also called calcium activated protease (CAP), but more commonly calcium activated factor (CAF) was first isolated as a phosphorylase B kinase activating factor (Huston and Krebs, 1968) but its function as a protease was not recognized.

Kohn (1969) detected calcium requiring proteolytic activity

having a pH optimum between pH 7 and 8 in studies of muscle homogenates. At about the same time Goll, Arakawa, Stromer, Busch and Robson (1970) discovered that rabbit muscle strips incubated overnight in calcium-containing solutions became extremely fragile. Microscopic examination of sections of the strips revealed the typical loss of Z-disc structure observed earlier in conditioned muscle. Control muscle strips incubated in EDTA failed to show similar changes. Subsequent investigation resulted in the isolation and characterization of CAF from porcine muscle (Dayton, Goll, Zeece, Robson, and Reville, 1976; Dayton, Reville, Goll, and Stromer, 1976). It was demonstrated that the purified enzyme could mimic the changes observed during conditioning of muscle fibers. When myofibrils were irrigated with CAF, the Z-discs could be observed to disappear.

CAF was shown to have a pH optimum of 7.5 using both myofibrils and casein as substrates. The enzyme requires 1mM Ca^{2+} for optimal activity and also the presence of a reducing agent (2-mercaptoethanol or dithiothreitol). CAF was irreversibly inhibited by alkylation with iodoacetamide. The later studies of Ishiura, Murofushi, Suzuki, and Imahori (1978) using chicken CAF revealed three 2-nitro-5-thiobenzoate titratable cysteine residues per mol of CAF in Ca^{2+}-free buffer. On the addition of Ca^{2+}, one more cysteine became titratable. Two additional residues were titrated after denaturation. Hence Ca^{2+} is necessary to activate an essential cysteine residue at an active site.

Dayton's purified porcine CAF has a sedimentation equilibrium molecular weight of 112,000 daltons but migrates as two subunits with molecular weights of 80,000 daltons and 30,000 daltons on SDS gels. Dayton et al. (1975) believed the 30,000 dalton subunit to have a regulatory function.

However, Ishiura et al. (1978) did not find the 30,000 subunit in their preparations from chicken and regarded it as a contaminant of the isolation procedure.

To determine the substrate specificity of chicken CAF, Ishiura et al. (1980) analyzed fragments of oxidized insulin B chain after hydrolysis by CAF. Their results indicated that CAF showed no preference for any specific amino acid sequence, but rather that the preferred site of hydrolysis was near the center of the long peptide chain, with other less preferred cleavage sites occurring closer to the ends of the substrate. Since CAF would hydrolyse none of the thirteen amino acid esters or small peptides commonly employed in protease assays, it was concluded that CAF only hydrolyses proteins. CAF would, however, only hydrolyse hemoglobin, myoglobin, cytochrome-C, bovine serum albumin, ovalbumin, phosphorylase and calmodulin if these proteins were denatured. Few non-muscle proteins were hydrolysed in their native form.

Because CAF was the first enzyme that was shown to degrade myofibrils at neutral pH, its effects on the myofibril proteins were studied with great interest, in an attempt to explain the physiological effects observed in electron micrographs. When entire myofibrils are treated with CAF, several changes are observed (Dayton, Goll, Reville, Zeece, Stromer, and Robson, 1974). Tropomyosin and troponin are degraded. At the same time a 30,000 dalton product of hydrolysis appears. α-actinin is released from the Z-disc matrix. C and M protein are also degraded, but to a lesser extent. Using purified troponin, a 30,000 dalton component was shown to be a product of the hydrolysis of TN·T (Dayton et al., 1974; Ishiura, Sugita, Nonanka, and Imahori, 1979).

It was of considerable interest that CAF caused the solubilization

of α-actinin from the Z-disc. Since CAF removes Z-discs, and since α-actinin is probably bound to thin filament, it was expected that CAF would degrade α-actinin and/or actin. However, CAF affected neither the molecular weight of actin or α-actinin, nor altered the N and C terminal residues of either protein (Dayton et al., 1975). Therefore, CAF did not degrade α-actinin or actin.

The degradation of C protein, M protein, tropomyosin and troponin, could explain the electron microscope observations of alterations in the structures that contain these proteins. However, the effects on the Z-disc remain unexplained.

Dayton et al. (1975) proposed a mechanism in which CAF was assigned the role of initiation of degradation of the myofibril. This has implications for the cellular role of CAF in the mechanism of myofibril turnover. Several lines of evidence support the idea that CAF is instrumental in such myofibril catabolism.

Cellular protein maintenance involves a delicate balance where protein synthesis exactly balances the elimination of protein that has outlived its usefulness. In radiolabelled amino acid incorporation studies, it was shown that the different muscle proteins exhibit a wide range of half lives (Funabiki and Cassens, 1972; Low and Goldberg, 1973). In muscle atrophy due to a disease such as muscular dystrophy, or due to denervation, marked changes in the Z-disc structure occur. These include hypertrophy, streaming, disintegration and disappearance (Engel, 1968). These observations, when coupled with the fact that elevated levels of CAF exist in atrophying muscle (Kar and Pearson, 1976; Kohn, 1969) imply that CAF may be involved in the destruction of both myofibrils and Z-discs in degenerating muscle tissue.

Dayton et al. (1975) speculated that CAF may initiate myofibril breakdown, because CAF hydrolyses the C and M proteins and troponin, and disrupts the Z-disc structure via an unknown mechanism. By hydrolysing the components thought to stabilize the thick and thin filaments, and by disrupting the Z-disc, complete myofibril disaggregation might occur.

However, disruption of the entire myofibril has never been observed. It is now recognized that the Z-disc is the primary site of CAF activity. C and M proteins are not hydrolysed as readily; changes in the C and M lines occur chronologically much later and are less pronounced than those in the Z-disc (Dayton et al., 1975). In addition, alterations in the troponin system are not likely to result in overwhelming degradation of the thin filament as troponin merely serves a regulatory function. The recent report that CAF has been located primarily at the Z-disc by fluorescent antibody techniques (Ishiura et al., 1980) also confirms the site of CAF activity. Thus the observed Z-disc hydrolysis by CAF is most likely responsible for myofibril fragmentation and the development of tenderness during conditioning.

The protein desmin is also hydrolysed by CAF (Hubbard and Lazarides, 1978). The location of desmin filaments at the periphery of the Z-disc may not directly cause the observable changes in the density of the Z-disc. However, since the desmin filaments encircling the Z-discs are responsible for the organization of the Z-discs, the hydrolysis of desmin may be responsible for another Z-disc-related alteration. In conditioned muscle the Z-discs are often observed out of register (Suzuki, Saito, Iwai, and Nonami, 1978; Suzuki, Saito, Sato, and Nonami, 1978). Thus the hydrolysis of desmin by CAF may be responsible for disrupting

the Z-disc connections between adjacent filaments.

Statement of the Problem

It is apparent from examination of the literature concerning the Z-disc, that many of the observed changes in its structure during post-mortem aging require further explanation. The importance of the Z-disc as the key structure which provides continuity and strength to the myofibril cannot be understated. Its weakening seems to account for the fragility that develops in post-mortem muscle with conditioning.

Since the enzyme CAF is the only detectable degradative enzyme active against myofibrillar components in post-mortem muscle, and since its effects on muscle ultrastructure and proteins resemble those observed in post-mortem aging, this research was undertaken in an attempt to expose information which could lead to a fuller understanding of the relationship between the mode of action of CAF and degradation of the Z-disc. The question posed then, is this: "How does CAF contribute to the breakdown of the Z-disc?"

Chapter 2

MATERIALS AND METHODS

PREPARATION OF PROTEIN SAMPLES

Minced Muscle

Chicken breast muscle, including both the pectoralis major and minor from six to ten week old broilers (Lilydale Poultry Farms, Edmonton) was excised immediately following exsanguination and chilled on ice. The excised muscle was trimmed free of visible connective tissue and fat, then finely minced in a Moulinex food processor (Moulinex Corp., France) for thirty seconds. All subsequent preparations were performed at 0 - 2°C unless otherwise specified.

Myofibrils

Using a Waring blender (Waring Products Co.), minced muscle was suspended using three - ten second bursts in five volumes of:

(a) 0.25 M sucrose, 0.05 M Tris-hydroxymethyl aminomethane (Tris)-HCl pH 7.6, 1mM ethylenediaminetetraacetic acid (EDTA).

The suspension was stirred for one hour then centrifuged for ten minutes at 2500 xg in the JA-14 rotor of a Beckman J-21 centrifuge. The pellet was again extracted by stirring for one hour in solution (a), filtered through two layers of cheesecloth, then extracted for ten minutes in each of the following solutions:

(b) 0.05 M Tris-HCl, 1mM EDTA, pH 7.6

(c) 0.15 M NaCl, .03 M Tris-HCl, pH 7.6

(d) 1mM EDTA

(e) distilled deionized water

and was finally washed by resuspension three times in:

(f) 0.15 M NaCl, 0.03 M Tris-HCl, 1mM NaN_3, 0.1mM EDTA, pH 7.6

and stored at 2°C in the same solution.

Isopycnic Centrifugation of Myofibrils

Myofibrils prepared by sedimentation were further purified by isopycnic centrifugation in a discontinuous sucrose gradient following a modification of the procedure of Maruyama, Matsubara, Natori, Nonomura, Kimura, Ohashi, Murakami, Handa and Eguchi (1977). A discontinuous sucrose gradient was prepared consisting of 9 ml of 68% sucrose, 14 ml 55%, 7 ml 25% and 10 ml 10%, all containing 0.15 M NaCl, .03 M Tris-HCl, 1mM NaN_3 and 0.1 mM EDTA pH 7.6. Five ml of myofibrils (20 mg/ml) were layered on the surface of each gradient and centrifuged in a Beckman SW 27 rotor at 130,000 xg for twelve hours. Myofibrils formed a single layer at the interface of the 55% and 68% sucrose layers. No pellet of whole cells or debris was evident.

Preparation of Myofibril Ghosts

A suspension of prepared myofibrils was sedimented at 2,500 xg. The pellet was then resuspended and extracted three times by stirring in five volumes of modified Hasslebach-Schneider solution (H·S) containing 0.6 M KCl, 0.01 M $Na_4P_2O_7$, 0.1 M sodium phosphate buffer, 1mM $MgCl_2$, pH 6.4. After each one-hor extraction, the suspension was centrifuged at 9,800 xg for fifteen minutes. The resultant pellet was then extracted three times for one hour each time, using five folumes of potassium iodide solution

(KI) containing 1.0 M KI, 10mM $Na_2S_2O_3$, 10mM NaH_2PO_4, 10mM ATP pH 7.5. The insoluble residue was then washed four times by stirring in five volumes of distilled water and gently centrifuging the suspension at 500 xg for five minutes. The sticky residue was stored at 2°C in distilled water containing 2mM NaN_3.

H·S and KI-Soluble Proteins

Soluble protein from the first H·S or KI extractions was prepared by dialysing approximately 100 ml of the supernatant solution from the appropriate extraction solvent against four or five changes of 3.5 litres of 0.1M NaCl, 0.05M Tris-HCl pH 7.6. Precipitated protein was dispersed using a teflon ball homogenizer. The entire suspension was used in CAF assays. Residual KI was detectable at concentrations greater than 50 μM by the following assay: Five drops each of two solutions containing a) 0.2M $K_2Cr_2O_7$, in 1M HCl and b) 0.8% (w/v) soluble starch were added to a 2 ml protein sample and allowed to stand ten minutes. KI at concentrations greater than 50 μM was detectable by a colour change from yellow to green. The presence of 50 μM had no significant effect on CAF activity.

Dialysis

Dialysis tubing (Fisher Scientific, Pittsburgh, PA) was prepared by boiling for ten minutes in 10 mM $NaHCO_3$. After rinsing in distilled H_2O, tubing was stored at 2°C in 10mM NaN_3.

Aged Muscle

Chicken breast muscle used in aging experiments was stored in a plastic bag wrapped in paper towelling soaked in 2 mM NaN_3 to retard bacterial growth. One to two-gram muscle samples were cut from the

interior of aged samples, accurately weighed, and homogenized for thirty seconds at high speed in a Sorvall omni-mixer (Sorvall Inc., Newtown, Conn.). Myofibrils were prepared and subsequent extraction was performed as previously described for fresh muscle.

Smooth Muscle Contractile Protein

The mucosa and connective tissue were removed from fresh chicken gizzard, and the remaining tissue was finely minced and extracted as described for preparation of myofibrils. Subsequent H·S and KI extraction was also identical.

Actin Purification

Actin was purified from an acetone powder by a modification of the method of Straub as described by Rees and Young (1967). Chicken breast muscle acetone powder was prepared by the method of Seraydarian, Briskey, and Mommaerts (1967). Approximately 100 g fresh chicken breast muscle was coarsely minced, suspended in 3.3 volumes of Guba-Straub solution (0.3 M KCl, 0.09M KH_2PO_4, 0.06M K_2HPO_4) and stirred for fifteen minutes. 13.3 volumes deionized cold H_2O was added and the suspension was strained through two layers of cheesecloth. The insoluble residue was then added to four volumes of 0.05M $NaHCO_3$, stirred for twenty minutes, and again strained. The residue was again extracted with 0.05M $NaHCO_3$ for twenty minutes, strained, then added to one volume of 0.05M $NaHCO_3$, 0.05M Na_2CO_3 and stirred ten minutes. Ten volumes of 50 μM $CaCl_2$ was then added to the suspension and stirred for ten minutes more. The strained residue was washed twice with ten volumes of cold deionized water, then three times for five minutes each with three volumes of cold acetone. The residue was spread on filter paper and air dried overnight at room

temperature. Yield: 3.6 g.

3.6 g acetone powder was suspended in 65 ml 0.5mM ATP, 0.5 mM 2-mercaptoethanol, 0.2mM $CaCl_2$ pH 7.5 (ATP solution) and stirred for thirty minutes. The suspension was centrifuged at 9,800 xg for thirty minutes. The supernatant solution was further clarified by filtration through an 8μ millipore filter. The soluble G-actin was polymerized to F-actin by adding KCl to 0.1M and $MgCl_2$ to 1mM. Polymerization was allowed to take place at room temperature for two hours. The polymerized F-actin was collected by centrifugation in a Beckman Type 40 rotor at 140,000 xg for two hours. The transluscent pellet was resuspended in 3.5 ml of the ATP solution then dialysed overnight against three changes of 250 ml of the same solution. The resultant G-actin solution was clarified by repeating the centrifugation in the Type 40 rotor then applied to a 2.2 x 46 cm G-200 (Pharmacia) column and eluted in the same buffer at a flow rate of 2.5 ml/hour and collecting 2.5 ml fractions. The single protein peak was eluted and fractions were pooled. Twenty μg of the material was applied to a gel. The protein migrated as a single band. This was G-actin. The F-actin used in some experiments was formed by polymerization of G-actin as described previously.

ASSAY OF PROTEOLYTIC ACTIVITY

Assay of CAF Activity

Proteolytic activity of CAF was determined in standard incubation mixtures that contained:

0.1M NaCl

0.24 percent (w/v) alkali-denatured casein (prepared fresh prior to use)

5mM 2-mercaptoethanol

5mM $CaCl_2$ or EDTA

0.1M Tris-acetate buffer pH 7.8 and

0 to 2 mg protein containing CAF activity

in either 0.5 or 1.0 ml total volume. Assays more than three hours in duration contained 2mM NaN_3. To ensure that the activity observed was due to CAF, three controls were run with each assay: (a) no enzyme (a CAF blank solution was substituted), 5mM EDTA; (b) no enzyme, 5mM $CaCl_2$; (c) CAF added, 5mM EDTA. All reactions were incubated in a water bath at 25°C.

Reactions were stopped by the addition of 100mM EDTA to a final concentration of 10mM, and the protein precipitated by the addition of an equal volume of ice cold ten percent trichloroacetic acid (TCA). Complete precipitation was allowed to occur for one hour at 0°C then tubes were centrifuged at 3,000 xg for five minutes. The absorbance of the supernatant fluid was read at 280 nm against the EDTA blank.

One unit of CAF activity was expressed as the amount of enzyme which catalyzed an increase in absorbance at 280 nm (ΔA_{280}) of 1.0 unit/ml per hour under these standard assay conditions. In all experiments, the amount of CAF added was expressed in units of activity. Since the specific activity in CAF preparations varied with the age of the preparation, and since in some cases pure preparations of CAF were not prepared (i.e., in assays where beef CAF was used) units of activity most accurately expressed the quantity of active enzyme added to incubation mixtures.

In some experiments, myofibrils (12 mg/ml) or ghosts (6 mg/ml) were substituted for casein in reaction mixtures. Denatured myofibrils or KI soluble protein used as substrates were denatured under the follow-

ing conditions:

(a) in 0.1M HCl for three hours at 25°C

(b) in 0.1M NaOH for three hours at 25°C

(c) in 8M urea for three hours at 25°C

(d) by heating in a boiling water bath for five minutes.

All samples were subsequently dialysed against 0.1M NaCl, 0.05M Tris-acetate pH 7.5 before incubation with CAF.

In assays of beef CAF activity on beef myofibrils, reactions were stopped using EDTA, and the ΔA_{280} of the supernatant was measured against appropriate blanks without precipitation with TCA.

In experiments where H·S and KI extraction followed incubation with CAF, after EDTA was added to stop the enzyme activity, an appropriate amount of EDTA or $CaCl_2$ was added in order that each reaction mixture would contain identical amounts of EDTA and $CaCl_2$ prior to extraction.

In parallel extractions of CAF-treated or aged myofibrils, and controls, the experimental (i.e., CAF + Ca^{2+}) mixture often differed in protein concentration from control extracts or residues because of the CAF activity. In order to facilitate direct comparison, identical volumes were used for preparation of samples for SDS-PAGE.

Hammarsten Casein

Hammarsten casein used in the routine assay of CAF activity, was prepared by four cycles of isoelectric precipitation of commercially available casein. Forty g of casein (Fisher, vitamin-free) was dissolved by stirring in two litres of water. The pH was maintained at pH 7 to 8 by dropwise addition of 10M NaOH. After filtration of the suspension the pH was slowly adjusted to pH 4.6 using 0.1M HCl. After the precipi-

tate settled, the supernatant solution was decanted, and two litres more of water were added and the pH readjusted and maintained between pH 7 and 8. Insoluble material was removed by filtering through glass wool. Casein was precipitated and resuspended three more times and finally dialysed against four litres distilled water, then lyophilized.

Alkali-Denatured Casein

Sixty mg casein was dissolved in five ml 0.1M NaOH and heated in a boiling water bath for five minutes. The cooled solution was neutralized using 1M HCl. This casein solution was then combined with the other ingredients of the CAF assay mixture.

Protein Concentration

Protein was quantitated by the biuret method of Gornall, Bardawill, and David (1949) or by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin (Sigma, St. Louis, MO) as a standard.

POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE

Sodium dodecyl sulfate (SDS) gels were prepared using the system of Porzio and Pearson (1976) for muscle proteins. This method uses a ten percent acrylamide concentration and an acrylamide to bis-acrylamide ratio of 100:1 coupled with low current (1 ma per tube) to achieve optimal protein band separation.

Acrylamide was recrystallized from chloroform, dried under vacuum and stored at 2°C. Electrophoresis grade bis-acrylamide was obtained from Bio-Rad Laboratories (Richmond, CA). Tetramethylethylenediamine

(TEMED) was redistilled. The other chemicals were reagent grade or better.

Preparation of Gels.

The acrylamide gels were prepared by mixing the following volumes of stock solutions:

18 ml 25% acrylamide, 0.25% bis-acrylamide (w/v)

9 ml 2.0M Tris-glycine buffer (0.5M Tris, 1.5M glycine, pH 8.8)

4.5 ml 60% glycerol (v/v)

1.8 ml 2.5% SDS, 2.5mM EDTA

1.8 ml 1% TEMED

1.8 ml 1% ammonium persulfate and

8.1 ml distilled H_2O

The TEMED was added last, and the solution gently stirred just before loading 2.3 ml into each of eighteen glass tubes (6 mm x 110 mm). Each aliquant was carefully overlaid with 400mM Tris-gly pH 8.8, 0.04% TEMED, 0.1% SDS and .004% ammonium persulfate. The gels were allowed to polymerize in the dark for several hours before use.

Gels were then mounted in a Buchler disc-gel apparatus (Buchler Industries, Fort Lee, NJ). One litre of running buffer containing 0.2M Tris-glycine pH 8.8, and 0.1% SDS was divided between the upper and lower chambers. Protein samples containing glycerol were gently layered on top of each gel using a Hamilton syringe (Hamilton Co., Reno, Nev.). The proteins were run into the gels by applying a low current of 0.25 ma per tube for thirty minutes. Then current was increased and the gels were run at 1 ma per tube for seven hours. The chambers were cooled by running tap water. The gels were then stained for twelve hours using a solution of two percent Coomassie Brilliant Blue in fifty percent ethanol and ten

percent acetic acid. Gels were partly destained for fifteen minutes in a Canalco (Canalco Corp., Rockville, MD) electrophoretic destainer using five percent isopropanol and 7.5 percent acetic acid. The gels were then stored in seven percent acetic acid. Destaining was completed by warming to 40°C for twenty-four hours. A record of the protein bands was kept by photographing the gels using Kodak Panatomic·X film for maximum detail and an orange filter for enhanced contrast.

Protein Sample Preparation

Protein samples containing from 0.25 mg to 5 mg in 0.5 ml were diluted 1:1 with a solution of 2.5% SDS, 2.5mM EDTA; 10 µl of 2-mercapto-ethanol were added to each millilitre. Samples were heated in a boiling water bath for five minutes, cooled, then diluted 1:1 using sixty percent glycerol. Sample volumes of two to two hundred µl containing three to one hundred and fifty µg protein were typically applied.

Densitometry of Stained Gels

Stained gels preserved in seven percent acetic acid were scanned in the same solution for the accurate determination of relative mobility (RM) and for the measurement of relative protein concentrations.

Gels were scanned in a Gilford 2520 gel scanning attachment at a wavelength of 560 nm using a slit plate of 0.10 x 2.36 mM, scanning at 2 cm/min.

Peak areas used for determining relative protein concentration were measured using an OTT Planimeter (Burrel Corp., Pittsburgh, PA). By applying increasing concentrations of myofibril protein to a series of gels, it was determined using the actin band that peak area was directly proportional to the amount of protein applied in the range between 0. to

3.0 A_{560} units. Quantitative comparison of protein between gels was only made between gels that had been stained and destained in the same batch.

Molecular Weight Determination

The molecular weights (MW) of myofibril proteins were determined by mixing them with protein standards of established molecular weight. After electrophoresis, the RM of each protein (measured on the densitometer trace) was compared to the mobility of standard proteins in the same gel, and molecular weights determined from a reference plot of Log MW versus R Mobility. Actin was arbitrarily assigned a RM of 1.0. All other protein mobilities were compared to that of actin.

Preparative SDS Gel Electrophoresis

For the purification of actins from mixtures of KI soluble proteins, or from KI insoluble residue, a one-step electrophoresis resulted in a pure protein preparation.

Acrylamide gels were prepared as previously described in this section. Usually two gel devices were run in series. Thus, thirty-six gel tubes could be run at the same time.

Samples were prepared as described previously, except that protein concentrations were increased. It was found by trial and error that up to 400 µg KI soluble protein could be applied to each gel in a volume of 50 to 100 µl. However, samples of ghost protein had to be much more dilute, 100 µg was the maximum that could be applied in a volume of 200 µl.

Electrophoresis was run for 9.5 hours at 1.25 ma/tube. Under these conditions, the actins migrated about seventy-five percent the length of the gel and were sufficiently separated from any other pro-

tein bands detectable by scanning at 280 nm or by staining with Coomassie blue. After gels were removed from the tubes, they were immediately scanned at 280 nm. The cuvette was one third filled with electrophoresis chamber buffer, the gel was inserted, and scanned at 280 nm using a 0.20 x 2.36 mm slit plate, and scanning at 2 cm/min.

When the actin passed through the light path the gel transport mechanism was stopped and the gel was nicked at that spot using a sharp scalpel. Each gel was then removed from the cuvette and the actin band was cut out and stored at 2°C.

Final Purification of Proteins From Gel Slices

The elution of the protein from gel slices was performed after the method of Lazarides (1977). A disposable 10 ml pipet was fitted with a piece of dialysis tubing knotted about 3 cm from the tip to seal the bottom of the pipet. The pipet was then half-filled with SDS running buffer (0.1% SDS, 0.05M Tris-acetate, pH 7.8) and a "Kimwipe" tissue plug inserted into the tip of the pipet. The rest of the pipet was filled to capacity with coarsely chopped gel slices, then filled with buffer, and plugged with another tissue plug. Care was taken to eliminate any air bubbles from the pipet.

Pipets were then inserted into an electrophoresis apparatus with the lower solution covering two thirds of the dialysis membrane. The upper chamber was filled with buffer as well, and the protein was eluted for twenty-four hours with an applied voltage of 120 V. After elution, the dialysis bag containing the protein in SDS running buffer was removed.

SDS was separated from the protein, using a modification of the procedure of Weber and Osborne. Small columns (2 ml packed volume) of

Dowex AG1-X2 (200-400 mesh) were prepared in Pasteur pipettes and pre-equilibrated with .05M Tris-acetate buffer, pH 7.8. Each protein sample was passed through a column and about 2 ml fractions were collected. Approximately eighty percent of the total UV (280 nm) absorbing material was recovered. The yield from 144 gel tubes containing ghost protein was 7.6 mg. Ninety gel tubes of KI soluble protein yielded 2.8 mg (protein determined by Lowry).

Isoelectric Focusing

Isoelectric focusing (IEF) was performed using 7.5 percent (40:1, acrylamide: bis-acrylamide) gels in 8M urea. Reagents were prepared as previously described. 10M urea stock solution was stored containing a small amount of Rexyn I-300 ion exchange resin to remove decomposition products. Prepared gels contained 7.5% (w/v) acrylamide, .188% (w/v) bis-acrylamide dissolved in 8M urea, 5% (v/v) glycerol .05% TEMED, .0125% ammonium persulfate and contained 1.4% pH 5-7 ampholytes and 0.6% pH 2-11 ampholytes (Bio-Lyte, Bio-Rad Laboratories). Protein samples were dissolved in 10M urea and added to the gel mixture prior to polymerization.

The lower (anode) chamber solution consisted of 0.02N H_2SO_4. The upper chamber was filled with 0.02N NaOH. Proteins were focused for sixteen hours at 300 V then voltage was increased to 700 V for one additional hour to sharpen the bands. Gels were stained and destained as previously described.

AMINO ACID ANALYSIS

Eight hundred to nine hundred μg of KI-soluble actin and ghost actin purified by preparative SDS-PAGE were used for amino acid analysis.

Protein samples were hydrolysed in 6N HCl contained in evacuated ampoules at 110°C for twenty-four hours, then lyophilized and resuspended in sample buffer containing internal standards.

Analysis of hydrolysates was performed in duplicate on a Beckman 121 MB amino acid analyser, courtesy of the Department of Animal Science and calibrated with a Beckman calibration standard.

IMMUNOLOGICAL STUDIES

Preparation of Antisera

Samples of protein recovered from SDS-gels were dialysed against 0.1M NH_4HCO_3 then lyophilized. Samples containing 400 to 500 µg protein were dissolved in 400 µl 0.9 percent saline and added to an equal volume of Freunds' complete adjuvant (H37Ra, Difco) and emulsified.

0.1 ml samples were injected intra-peritoneally into each of a group of five BALB/cCr female mice aged eighty-eight days. Subsequently mice were reinjected with approximately 100 µg protein per mouse emulsified in Freunds' incomplete adjuvant administered by foot pad injection (50 µl/pad) at intervals of from four to six weeks.

Following five rounds of immunization, blood was collected by cardiac puncture. Blood was allowed to clot at 37°C for one hour then held at 2°C overnight. The serum was collected after centrifugation at 3,000 xg for ten minutes. The serum was diluted 1:1 using 0.9% saline and fractionated by adding ammonium sulfate to thirty-three percent saturation. After centrifugation the pellet was resuspended in one ml 0.9% saline and both the pellet and the ammonium sulfate supernatant solution were dialysed against three changes of 1.8 l of 0.9% saline.

Fractionated serum samples were tested for precipitating antibodies by double immunodiffusion in agar. Ten µl of each serum sample was placed in the centre wells of immunodiffusion plates (pattern C, Hyland) and challenged with ten µl containing 400 to 800 µg of protein isolated from SDS gels, or a control solution in surrounding wells. Diffusion was allowed to proceed at 2°C for twenty-four hours. Precipitating antibodies were detected only in the thirty-three percent ammonium sulfate-soluble fraction from mice immunized with ghost actin.

Indirect Immunofluorescence

Actin in glycerinated myofibrils was visualized by indirect immunofluorescence. Single glycerinated muscle fiber bundles were homogenized in one ml glycerol extracting solution for three to five minutes at high speed using the micro attachment of a Sorvall Omni-mixer. Drops of the resulting suspension were placed on coverslips and stored in a humidified chamber. After thirty minutes, the non-adherent myofibrils were rinsed off with Phosphate Buffered Saline (PBS) (137mM NaCl, 3mM KCl, 2mM KH_2PO_4, 8mM Na_2HPO_4, pH 7.5).

In some experiments, adherent myofibrils were then irrigated with the usual CAF assay solution containing 5mM EDTA or Ca^{2+} in the presence and absence of CAF and incubated ninety minutes at 25°C. Following incubation, coverslips were rinsed with PBS.

Coverslips were irrigated with 100 µl of the immunoglobulin fraction diluted 1:15 with PBS and incubated at 37°C for forty-five minutes. Coverslips were then rinsed twenty times by immersion in ten µl fresh PBS for two minutes each time.

Coverslips were then irrigated with 100 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG $F(ab')_2$ fraction (Polysciences

Inc., Warrington, PA) diluted 1:15, then incubated and subsequently rinsed as previously described. Slides were prepared by applying vaseline around the edge of each coverslip, then inverting them onto a glass slide.

Myofibrils were viewed using a Zeiss Photomicroscope III equipped with epifluorescence optics (Carl Zeiss, Oberkochen, Germany), courtesy of the Department of Zoology. Pairs of photographs of each microscopic field were taken using the 100x phase objective (N.A. 1.3). One photograph was taken using phasecontrast optics to identify zones within the myofibril. A second photograph of the same field was taken using the fluorescence optics to locate areas of FITC labelling. By comparison of the two photographs, fluorescent-labelled structures within the myofibril were identified. Ilford HP-5 film was exposed at ASA 1600 and overdeveloped fifty percent in Ilford Microphen. Exposure times were five to fifteen seconds.

ELECTRON MICROSCOPY

Glycerinated Fibers

Fresh chicken breast muscle was carefully teased into thin fiber bundles and tied at rest length to round wooden applicator sticks using silk thread. Fibers were preserved in a solution containing fifty percent glycerol, .137M NaCl, 2.7mM KCl, 9.6mM phosphate buffer (formulated from KH_2PO_4 and Na_2HPO_4) 4mM EDTA and 1mM NaN_3 pH 7.4., stored for one month at 2°C then at -15°C until required.

Electron Microscopy of Glycerinated Fibers

Prepared glycerol extracted fibers were incubated for ninety minutes at 25°C using the usual CAF assay mixtures containing (a) 5mM

$CaCl_2$ only, (b) 5mM EDTA and 0.7 units/ml CAF, and (c) 5mM $CaCl_2$ and 0.7 units/ml CAF. Some fibers were then extracted three times using H·S solution for fifteen minutes at a time. Thereafter, some were further extracted three times in KI solution for fifteen minutes at a time. Following extraction or CAF incubation, fibers were washed at least three times in .15M NaCl, .03M Tris-HCl, 1mM NaN_3, 0.1mM EDTA.

Fibers were then rinsed once with .05M cacodylate buffer pH 7.2. Samples were fixed in Karnovsky's fixative overnight, rinsed with cacodylate buffer and post-fixed in two percent OsO_4 for sixty minutes. After washing with H_2O, samples were dehydrated in a graded series of ethanol washes from fifty percent to ninety-eight percent then further dehydrated in two rinses in propylene oxide.

The fixed dehydrated fibers were then incubated in propylene oxide: Araldite 502 (3:2) overnight, then embedded by insertion into Araldite 502 - filled molds and cured for forty-eight hours at 65°C.

Thin sections were cut with a DuPont diamond knife onto Metaxaform Copper plain grids Mesh 300 (3.05 mm diameter). Sections were stained in uranyl acetate stain for fifteen minutes, rinsed with distilled water, stained with lead citrate stain for ten minutes, then washed again.

The sections were viewed using a Philips EM201, courtesy of the Department of Medical Bacteriology, and photographed on Eastman fine grain release positive film 5302.

Chapter 3

RESULTS

The experiments presented in this section were designed to closely investigate the effects of aging and of CAF on the proteins of the myofibril. The technique of SDS-PAGE was primarily used and is ideally suited (because of its reliability and resolution) to the qualitative and often quantitative analysis of the proteins present in complex mixtures such as those of the myofibril. Since it is well understood that CAF, by an as yet unexplained mechanism, is responsible for the degradation of the Z-disc and that the enzyme CAF is a proteolytic enzyme, it was likely that heretofore undetected proteolysis might be revealed by examination of the effects of CAF on the proteins of the myofibril in more detail. Because of the complex pattern of the protein bands in SDS-gels, it was thought possible that undetected changes produced by CAF activity might be revealed by fractionation of the protein constituents. The contractile protein solvents selected were Hasslebach-Schneider solution (H·S) used to extract crude myosin, then a KI solution used to depolymerize and extract soluble actin from the myosin-poor myofibrils. Following the extraction using these two solutions an insoluble residue remains, first identified by A. Szent Gyorgi in 1951 and called "ghost" myofibrils because of their striated microscopic appearance, despite

the extraction of the contractile filaments. The residue following such extractions contained primarily the proteins of the Z-disc.

The results that follow begin by characterizing the effects of CAF on the intact myofibril proteins and then confirm that those changes mimic the effects of aging. Then, using extracts of myofibrils, the proteolysis by CAF is more closely examined.

Chicken Breast Myofibrillar Protein

Myofibrils from fresh chicken pectoralis muscle were prepared by repeated cycles of sedimentation and resuspension of the insoluble protein to remove the soluble sarcoplasmic protein, and by filtration to remove large pieces of unhomogenized muscle and connective tissue. The SDS-polyacrylamide-electrophoretogram (after the method of Porzio and Pearson, 1976) of the proteins of the washed myofibrils from fresh muscle is presented in Plate 1a and is accompanied by the corresponding densitometer scan. The major myofibrillar proteins and their size in order of descending subunit molecular weight are indicated. The molecular weights were estimated by reference to standard proteins of known molecular weight which were added to myofibrillar protein samples (Figure 4). The identity of each of the major protein bands was assigned by comparison with established molecular weights of proteins for the chicken myofibril. A complete summary of the proteins of the chicken myofibril assembly is presented in Table 1 and includes the molecular weights determined in these studies using this gel system for comparison with other reported values. In Plate 1 it can be seen that the major proteins were easily resolved by this procedure. In most acrylamide gel formulae the band resolution deter-

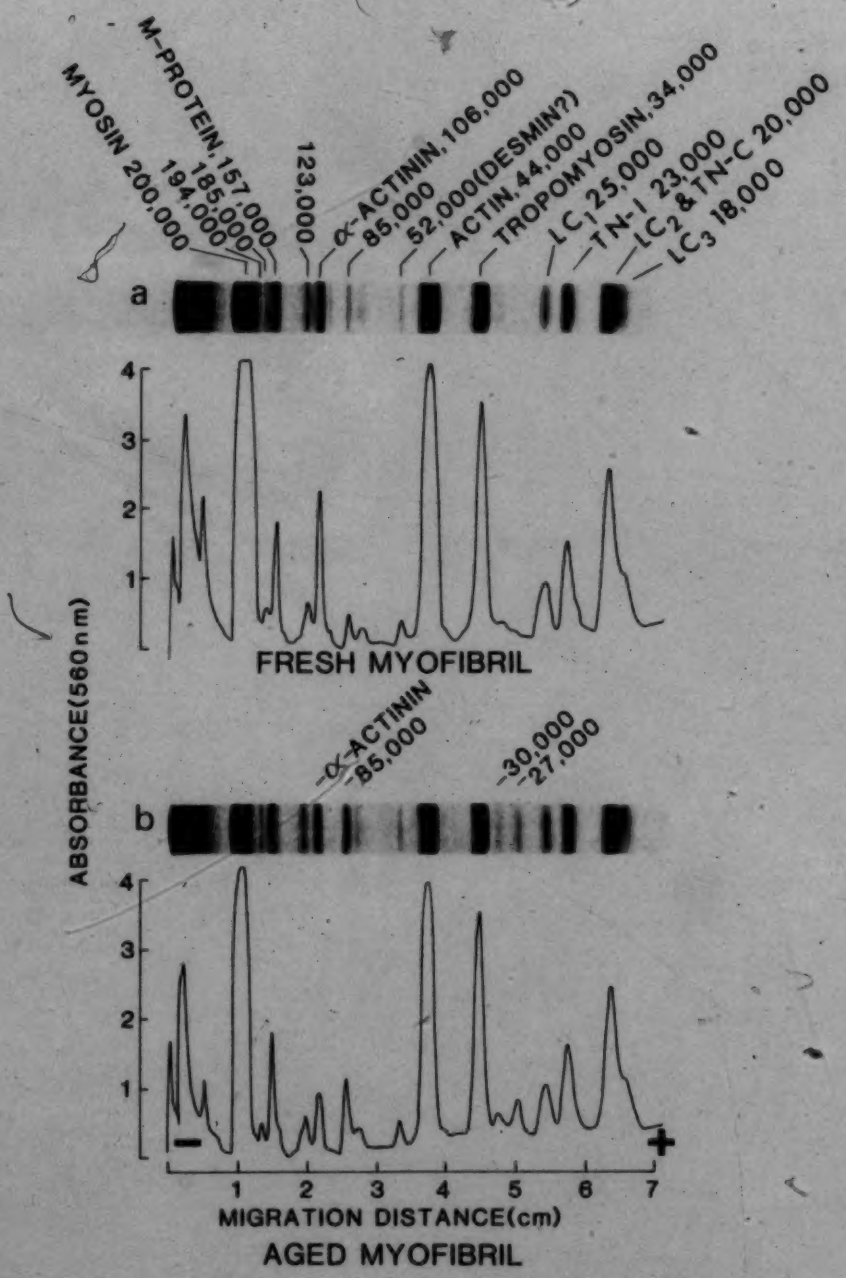

Plate 1. Densitometry of SDS-PAGE of myofibrils from fresh and aged chicken pectoralis muscle. Samples containing 100 µg myofibril protein from (a) fresh muscle and (b) muscle aged two days at 0°C were applied to gels. Each gel is accompanied by its gel scan. The molecular weights of the proteins were determined by comparison of the RM of each band with the protein standards in Figure 4. Tentative identity of the proteins was established by comparison of the determined molecular weights with established literature values.

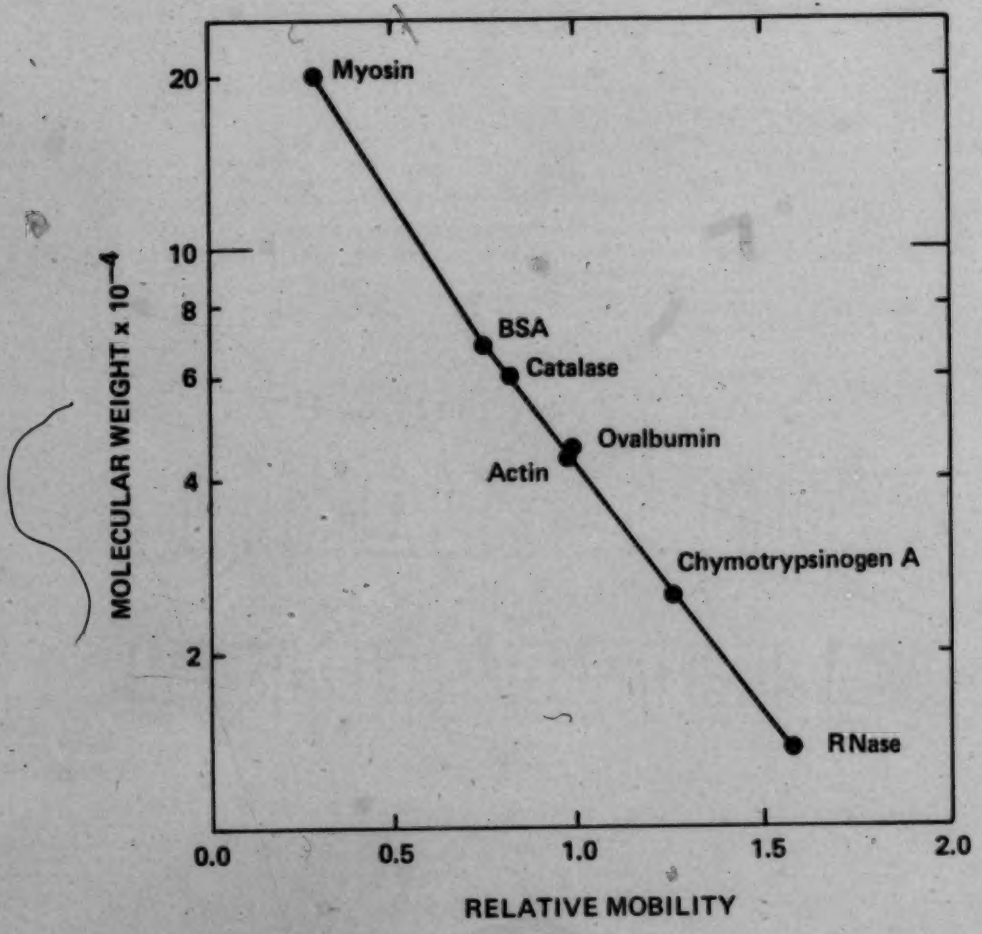

Figure 4. Molecular weight determination by SDS-PAGE. Mixtures of protein standards containing 3 µg BSA (68,000 daltons), 4 µg human catalase (60,000 daltons), 3 µg ovalbumin (48,000 daltons), 3 µg chymotrypsinogen A (25,000 daltons), 3 µg RNase (13,700 daltons) and 25 µg myofibril protein were applied to gels. Following staining gels were scanned and the relative mobility (RM) of each of the proteins in relation to that of actin (which was assigned an RM of 1.0) was plotted against the logarithm of molecular weight. A molecular weight of 200,000 daltons was used for myosin.

TABLE 1

Proteins of the Myofibril

Protein	Location in the Myofibril	Undissociated Protein	Subunit Weight	Source	Reference
Myosin	Thick filament	~ 460,000	200,000	Chicken	Rubenstein, Pepe, & Holtzer, 1977
Myosin LC_1	Thick filament		25,000	Chicken	Rubenstein et al., 1977
Myosin LC_2	Thick filament		18,000	Chicken	Rubenstein et al., 1977
Myosin LC_3	Thick filament		16,000	Chicken	Rubenstein et al., 1977
M-protein	M-line	165,000	165,000	Chicken	Strehler, Pelloni, Heitzman & Eppenberger, 1980
Creatine Kinase	M-line	88,000	44,000	Chicken	Strehler et al., 1980
C-protein	A-band	140,000	140,000	Rabbit	Offer, Moos & Starr, 1973
α-actinin	Z-disc	212,000	106,000	Rabbit	Suzuki, Goll, Singh, Allen, Robson, & Stromer, 1976
G-actin	Thin filament	42,780	42,780	Rabbit	Kuehl, Conti & Adelstein, 1975
Tropomyosin	Thin filament	32,600	32,600	Rabbit	Sodek, Hodges & Smillie, 1978

TABLE 1

(Continued)

Protein	Location in the Myofibril	Undissociated Protein	Subunit Weight	Source	Reference
Troponin-T	Thin filament		44,000	Chicken	Perry & Cole, 1974
Troponin-I	Thin filament	87,000	23,000	Chicken	Perry & Cole, 1974
Troponin-C	Thin filament		18,000	Chicken	Perry & Cole, 1974

iorates dramatically when more than 50 µg is applied. However, using the Porzio and Pearson technique, the bands are sharp and well defined even when loaded with 100 µg of protein. These gels illustrate that this technique is ideally suited for studies of both the major and minor protein constituents of the myofibril, many of which have only been tentatively identified as myofibril components.

The gel pattern in Plate 2b, obtained from the myofibrils prepared by isopycnic sucrose gradient centrifugation was identical to that obtained from myofibrils prepared by sedimentation alone (Plate 2a). This experiment confirmed that the washed preparations did not contain any proteins from easily sedimented whole cells or large cell fragments that might have been present in myofibrils prepared by sedimentation.

Aged Chicken Myofibrils

Myofibrils prepared from chicken pectoralis muscle aged for two days at 0°C exhibited some characteristic changes in their proteins which can be observed on gels. These changes indicated a limited and selective proteolysis.

The gel pattern of aged myofibrils is presented in Plate 1b. In this aged sample, the intensity of the α-actinin band has decreased, as indicated by the decrease in the height and area of the peak corresponding to that protein in the densitometer scan. In addition, two extra bands have appeared in the aged myofibril gel (having molecular weights of 30,000 and 27,000 daltons). An increase in the intensity of a protein band at 85,000 daltons was also observed. These results confirm the earlier observations of Hay et al. (1973).

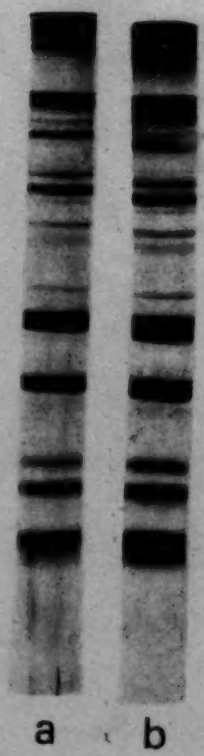

Plate 2. SDS-PAGE of myofibrils purified by sedimentation and by isopycnic centrifugation. 100 µg samples of myofibrils prepared by (a) sedimentation and purified further by (b) isopycnic centrifugation were applied to gels.

In similar experiments, additional changes were observed depending upon the time and temperature of aging. In Plate 3a, gels of myofibrils aged under differing conditions are shown for comparison. Myofibrils from muscle aged at 4°C for two days exhibited the characteristic changes of the 0° aged sample, and also revealed a decrease in the intensity of M-protein (158,000 daltons) and an increase in the band directly below it (150,000 daltons). After five days at room temperature (26°C), major degradation of TN-I as well as changes in high molecular weight proteins (160,000 to 200,000 daltons), including the degradation of myosin, were observed, and were accompanied by the accumulation of low molecular weight (MW) degradation products. These results show that during post-mortem aging of muscle, a wide variety of progressive degradative changes may occur.

The Effects of CAF on Isolated Myofibrils

The enzyme CAF has been considered to be responsible for many of the degradative changes that occur during post-mortem aging in muscle. CAF and purified myofibrils were incubated together at optimum conditions to characterize these effects. The gels prepared following incubation are presented in Plate 3b. The most prominent effects observed were the decrease in the intensity of the α-actinin band and the appearance of 30,000 and 27,000 dalton degradation products.

These results are compatible with those of Ishiura et al. (1979) and Yamamoto, Samejima, and Yasui (1979) who demonstrated the degradation of chicken TN-T into 30,000, 27,000 and 14,000 dalton products.

Plate 3. Comparison of SDS-PAGE of changes in myofibril proteins from aged muscle with changes in myofibrils incubated with CAF. (a) Myofibrils prepared from fresh muscle (0h), 100 µg, and from muscle samples aged 48 hours at 0°C, at 4°C and 144 hours at 26°C. (b) Myofibrils treated with CAF (0.7 units/ml final concentration) and 5 mM EDTA (-) or 5 mM $CaCl_2$(+) for one hour and 14 hours.

In chicken breast myofibrils, TN-T has a molecular weight of 44,000 daltons. Its presence (or disappearance due to hydrolysis) on gels is obscured by actin which also migrates with an apparent molecular weight of 44,000 daltons. The formation of a 30,000 dalton product of TN-T hydrolysis has also been reported in pork (Dayton et al., 1976; and in beef muscle (Olson et al., 1977; Penny and Ferguson-Pryce, 1979).

The decrease in the quantity of α-actinin has previously been shown to be a non-degradative phenomenon (Dayton et al., 1975) in that α-actinin remains unhydrolysed, but by some unexplained mechanism, is released from the myofibril matrix. The gels presented in Plate 4b confirm that α-actinin solubilized by incubation with CAF had the same molecular weight (106,000 daltons) as in the intact myofibrils.

In addition, several other less prominent changes can be seen in Plates 3b and 4a. These include the appearance of a band at 85,000 daltons, the solubilization of a small amount of myosin and two protein bands having molecular weights of greater than myosin (200,000).

Furthermore, when incubation time was extended (Plate 3b), degradation of the 157,000 dalton M-line component and of TN-I occurred, similar to that reported by Ishura et al. (1979) in chicken, and Dayton et al. (1975) in pork. Changes in the high MW region could also be seen.

The effects of CAF on isolated chicken myofibrils are similar in almost every respect to the effects of aging presented earlier. The changes observed in both cases involved initially the appearance of 30,000 and 27,000 dalton products, and a decrease in amount of α-actinin

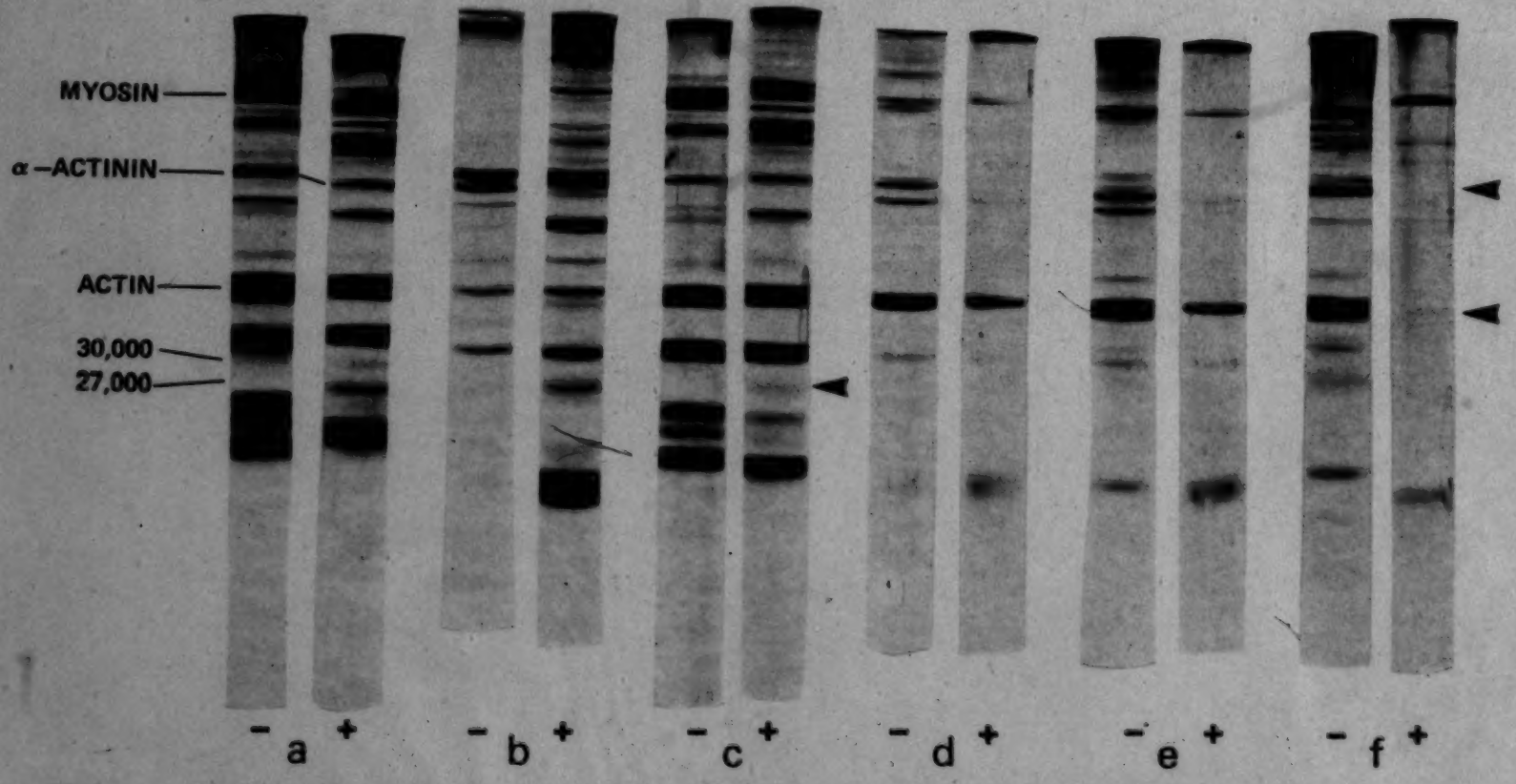

Plate 4. SDS-PAGE of myofibrils incubated for 14 hours with CAF prior to extraction with H-S and KI: protein distribution in extracts. Myofibrils (20 mg/ml) were incubated with CAF (1 unit/ml) and 5 mM $CaCl_2$ (+) or EDTA (-) for 14 hours at 25°C and subsequently extracted with H-S and KI. Gels depict: (a) myofibrils, 150 µg; (b) myofibril supernatant solution after incubation, 20 µg; (c) protein soluble in the first H-S extraction, 150 µg; (d) H-S insoluble residue, 50 µg; (e) protein soluble in the first KI extraction, 75 µg; (f) KI insoluble residue, 90 µg. The amount of protein is that applied to the control (-) gels with equivalent volumes applied to the experimental (+) gel to facilitate comparison.

remaining in the myofibril. In later stages, similar alterations in the troponins, M-protein and myosin were observed.

These results imply that CAF is responsible for the changes observed in the myofibril proteins during post-mortem aging. However, the effects of CAF on the proteins of the Z-disc and its observed microscopic structure remain only partially explained. It has been established that CAF releases the Z-disc protein α-actinin from the Z-disc. CAF also has been shown to decrease the Z-disc density of isolated myofibrils (Busch, Stromer, Goll and Suzuki, 1972). Muscle fibers incubated with CAF fracture at the Z-disc when homogenized, thus demonstrating the increased fragility at the Z-disc. These observed effects on the Z-disc cannot be explained by the hydrolysis of the troponins or M-line proteins as neither protein is Z-disc associated.

Fractionation of CAF-Treated Myofibrils

Because of the complex pattern of the myofibril proteins on SDS gels, the possibility existed that CAF hydrolysis of one or more of the less prominent protein components had gone undetected due to other overlapping protein bands. To further examine this possibility, CAF treated and control myofibrils were fractionated using H·S and KI, and the protein distribution in soluble extracts and insoluble residues was determined by SDS-PAGE.

The results of one such experiment are presented in Plate 4. The amount of protein solubilized in each extraction of the three control and CAF + Ca^{2+} incubations is presented in Table 2. In all

TABLE 2

Determination of Protein in Extracts of Control

and CAF Treated Myofibrils

		EDTA-Control (mg/%)*		Ca^{2+} Control (mg/%)		CAF + EDTA (mg/%)		Ca^{2+} + CAF (mg/%)	
1.	Initial protein	92.0	100.0	89.5	100.0	91.5	100.0	88.5	100.0
2.	Soluble protein after incubation	4.7	5.1	5.3	5.9	4.3	5.1	21.9	24.7
3.	Myofibril wash	1.9	2.1	2.4	2.6	1.9	2.0	8.3	9.3
4.	Total Soluble Protein Following Incubation (2 + 3)	6.6	7.2	7.7	8.5	6.2	7.1	30.2	32.0
5.	H·S Soluble Protein (first extraction)	44.9	48.8	45.8	51.2	44.2	48.3	33.1	37.4
6.	(second extraction)	11.8	12.8	11.5	12.8	10.6	11.6	10.4	11.7
7.	(third extraction)	1.3	1.4	2.8	3.2	1.0	1.1	1.8	2.1
8.	Total H·S Soluble Protein	58.0	63.0	60.1	67.2	55.8	61.0	45.3	51.2
9.	H·S Insoluble Residue	19.9	21.7	23.1	25.8	22.4	24.5	6.5	7.3
10.	KI-Soluble Protein (first extraction)	7.2	7.8	7.0	7.9	6.0	6.5	2.8	3.1
11.	(second extraction)	0.6	0.6	1.2	1.4	1.2	1.3	~0	~0
12.	(third extraction)	~0	~0	~0	~0	~0	~0	~0	~0
13.	First H_2O Wash	0.7	0.8	0.5	0.5	0.4	0.4	~0	~0
14.	Second H_2O Wash	0.2	0.2	0.2	0.2	0.3	0.3	~0	~0
15.	Total KI-Soluble Protein	8.7	9.4	8.9	10.0	7.9	8.5	2.8	3.1
16.	KI-Insoluble Residue	14.0	15.2	13.9	15.5	13.4	14.6	4.0	4.5
	Total Protein Recovery	87.2	94.8	90.5	101.2	83.2	91.2	82.3	90.8

* Data expressed in mg protein and as a percentage of the total initial protein.

cases, gels of the three controls were identical, only the CAF + EDTA gel is shown and represents the control (-) gels.

CAF treatment of myofibrils for fourteen hours resulted in the appearance of the 30,000 and 27,000 dalton bands (a) and in the solubilization of α-actinin. In addition to the appearance of an unidentified 85,000 dalton component and the appearance of low MW peptide fragments (b), changes were also observed in the high MW region of the gel and TN-I was degraded. The total protein solubilized by CAF + Ca^{2+} during the incubation was about thirty-two percent of the original myofibril protein. The seven to eight percent of the protein released in the control incubation consisted primarily of α-actinin.

The myofibril pellet was subsequently extracted with H·S solution. The pattern of the proteins solubilized appeared almost identical to that of the entire myofibril with the single exception that the extract contained proportionately less α-actinin (c). The other major myofibrillar proteins including myosin, M-protein, actin, tropomyosin, the troponins and myosin light chains were soluble. Since the other troponins were extracted, it is likely TN-T was also extracted as well and remains obscured in the actin band. The 30,000 and 27,000 dalton components were also soluble in H·S and appear in the CAF + Ca^{2+} gel. A trace of 85,000 dalton component also existed in this extract. Sixty-one to sixty-seven percent of the control myofibrillar protein was removed in the three H·S extractions. Only fifty-one percent was extracted in the CAF + Ca^{2+} incubation. The difference may be due in part to the proteolysis of troponins in that sample.

Following H·S extraction of the myofibrils, the control (-) residue (d) contained myosin, α-actinin, and actin. The CAF + Ca^{2+}

residue contained much less protein (7.3 percent in comparison with twenty-one to twenty-five percent in the controls), only myosin, little or no α-actinin and a reduced amount of actin were present.

The residue was further extracted with KI. An additional 8.5 to ten percent protein containing myosin, α-actinin and actin was removed (e). The final residue contained myosin, α-actinin, actin, the 123,000 and 85,000 dalton proteins, and low MW peptides (f) that constituted fifteen percent of the original myofibril protein.

The 123,000 and 85,000 dalton components were not present in all KI extracted residues. Since the two bands were only sometimes present in both fresh and aged samples, they were regarded as artifacts of the incubation or subsequent extraction of myofibril proteins.

The residue of the CAF + Ca^{2+} myofibrils (f) contained only myosin. Only 4.5 percent of the original myofibrillar protein remained. The other two proteins normally found in ghosts prepared by KI extraction (and found in the control incubations) are α-actinin and actin. This series of extractions has shown that α-actinin was solubilized first directly by CAF treatment and most of the remainder was removed in the subsequent H·S extraction. Most important, the actin that normally remains insoluble was absent. In the H·S insoluble residue (d), the amount of actin remaining was also reduced. These results indicated that the actin that normally remained in KI extracted myofibrils may have been removed by H·S extraction. However, there is no indication in the H·S extracted protein gels that more actin was extracted in the CAF + Ca^{2+} sample (c). The possibility existed that the small proportion of the actin that remained KI insoluble had been

hydrolysed by CAF.

The previous experiment demonstrates the possible effects of CAF on the proteins of the myofibril where proteolysis was allowed to continue at optimum conditions for an extended period of time. However, the extensive proteolysis observed in this model system (i.e., exogenous CAF plus purified myofibrils) may not truly reflect what occurs in muscle during normal post-mortem aging.

Myofibrils were therefore incubated with CAF for only one hour at 25°C prior to extraction. Results similar to those just presented were obtained. Plate 5 shows that the shorter one hour incubation produced only changes in the α-actinin and 30,000 and 27,000 dalton bands (a) with no degradation of myosin or M-protein or the appearance of the 85,000 band. Only α-actinin was solubilized by CAF treatment (b). This pattern was similar to the effects observed with aging at 0°C for two days (see Plate 1b).

In the H·S soluble protein, a similar pattern occurred as described previously, except no 85,000 band was present.

The H·S insoluble protein contained myosin, α-actinin, actin and tropomyosin. None of the extra bands in the α-actinin region of the fourteen-hour incubation were observed.

KI extraction of these residues removed some myosin, α-actinin, actin and TM (e).

The control residue, insoluble in KI (f), contained myosin, α-actinin, and actin. In the CAF + Ca^{2+} incubation, no α-actinin remained and the actin band that remained was considerably reduced in intensity.

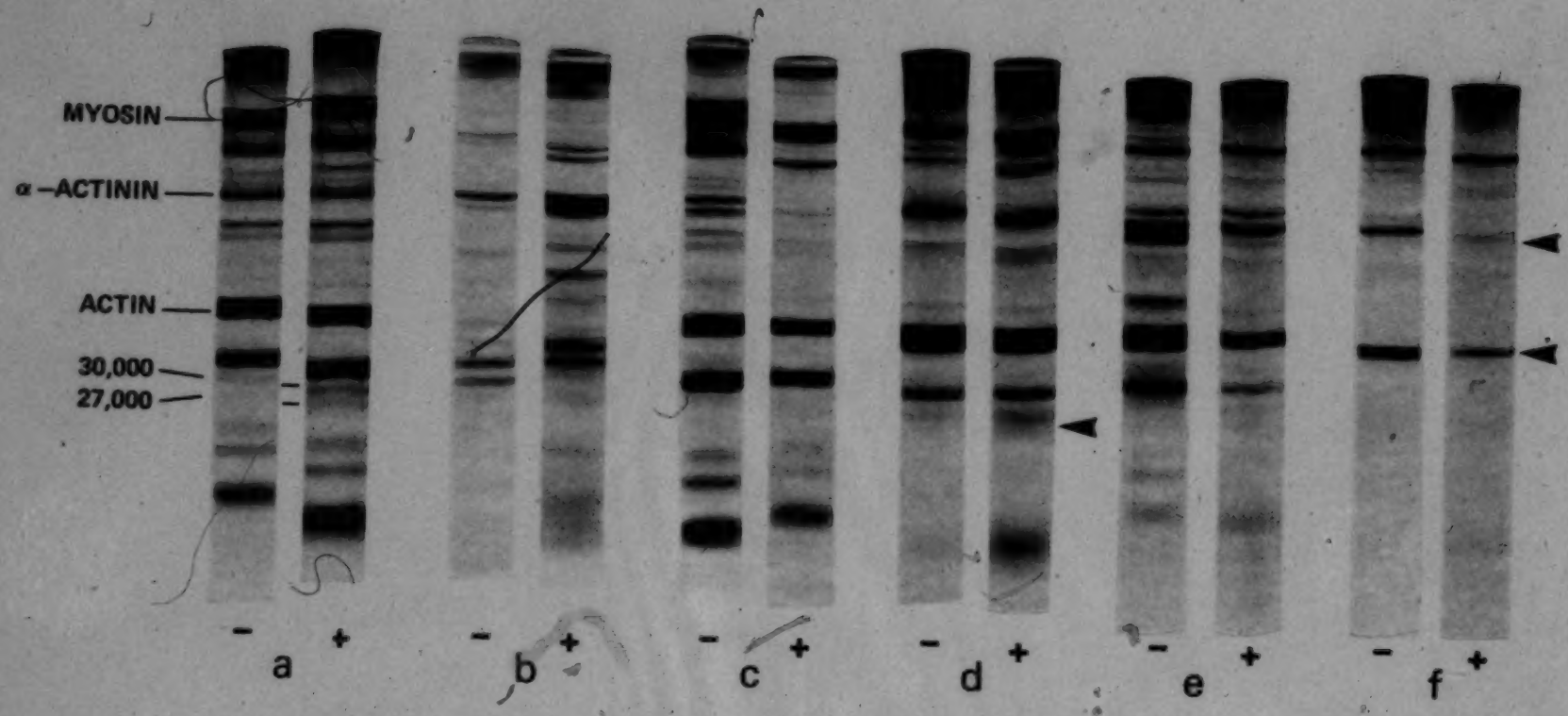

Plate 5. SDS-PAGE of myofibrils incubated one hour with CAF prior to extraction with H-S and KI: protein distribution in extracts. Myofibrils (12 mg/ml) were incubated with CAF (0.7 units/ml) and 5 mM $CaCl_2$(+) or EDTA (-) at 25°C for one hour, and subsequently extracted with H-S and KI as described in materials and methods. Gels depict (a) myofibrils, 100 µg; (b) myofibril supernatant solution after CAF incubation, 10 µg; (c) protein soluble in the first H-S extraction, 100 µg; (d) H-S insoluble protein, 100 µg; (e) protein soluble in the first KI extraction, 100 µg; (f) KI insoluble residue, 50 µg. The amount of protein represents that for the control (-) gel; equivalent volumes were applied to the experimental (+) gel to facilitate comparison.

These results demonstrated that even after as little as one hour of incubation with CAF, the amount of KI insoluble actin had been substantially reduced.

Extraction of Myofibrils From Aged Muscle

The previous experiments showed that KI insoluble actin had been reduced or completely eliminated by CAF in isolated myofibrils, depending upon the conditions of incubation. Could these changes be observed in normally aged post-mortem muscle?

Chicken breast muscle was aged for three days at 0°C. Myofibrils prepared from muscle samples were extracted as described previously. The extracted proteins were compared with unaged samples from the same muscle.

The results presented in Plate 6 show the decrease in α-actinin and appearance of the 30,000 and 27,000 dalton bands typical for aged muscle (a). The H·S soluble protein again has a similar appearance to entire myofibril protein but contains less α-actinin (b). The 30,000 and 27,000 dalton fragments were also soluble. KI extraction removes myosin, α-actinin and actin (c), though less α-actinin was evident in the KI extracts of aged muscle.

The KI insoluble ghosts of fresh muscle contained myosin, α-actinin, actin and a low MW component. In addition, small amounts of 85,000 and 123,000 dalton components could be observed. In aged ghosts, no α-actinin was detectable and the amount of actin had been substantially reduced. Measurement of the amount of residual actin in fresh and aged samples by densitometry revealed that only thirty-four

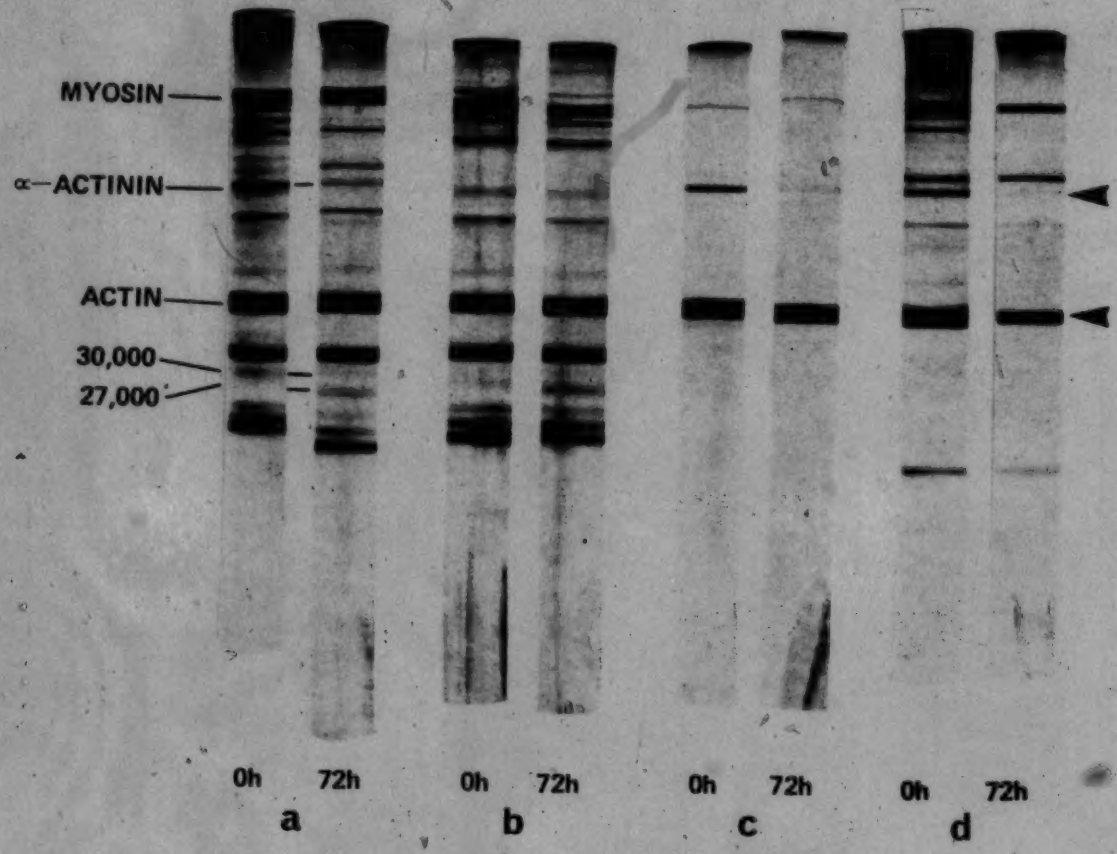

Plate 6. SDS-PAGE of myofibrils prepared from fresh and aged muscle, then extracted with H-S and KI: protein distribution in extracts. (a) myofibrils prepared from fresh muscle (0h) and muscle aged 72 hours at 0°C (72h), 100 µg; (b) protein soluble in the first H-S extraction, 100 µg; (c) protein soluble in the first KI extraction, 30 µg; (d) KI insoluble residue, 70 µg. Protein quantities represent those for the fresh (0h) sample; equivalent volumes were applied for the aged (72h) gels to facilitate direct comparison.

percent of the KI insoluble actin (in comparison with controls) remained in aged ghosts. In addition, the amount of myofibril protein remaining after KI extraction was reduced from about thirteen percent in fresh samples to about five percent in the aged samples. The densitometry and protein results were determined from triplicate samples from two experiments.

These results establish that the amount of KI insoluble actin in ghosts is reduced by aging, likely due to CAF activity in post-mortem muscle.

Hydrolysis of Extracted Myofibril Proteins by CAF

In order to determine if in fact KI insoluble actin was hydrolysable, and to further investigate the possibility that other myofibril proteins may be subject to CAF proteolysis, the extracted myofibril fractions were also treated with CAF (Plate 7). CAF treatment of entire myofibrils is again presented (a). In proteins extracted with H·S solution, little hydrolysis was observed (b). The 30,000 and 27,000 dalton component appeared, most likely as a result of the hydrolysis of TN·T.

The CAF-treated H·S residue (c) showed the development of only a 27,000 dalton component and low MW degradation products. However, in the CAF treated KI-residue (e), near complete hydrolysis of all components occurred. In comparison, the KI soluble proteins (d), both myosin and α-actinin were hydrolysed, but actin remained completely intact. It had been previously reported that the structure of myosin is particularly susceptible to denaturation in KI. Here, myosin was degraded by CAF to three fragments, predominantly a 150,000 dalton fragment, likely the same product observed in the effects of

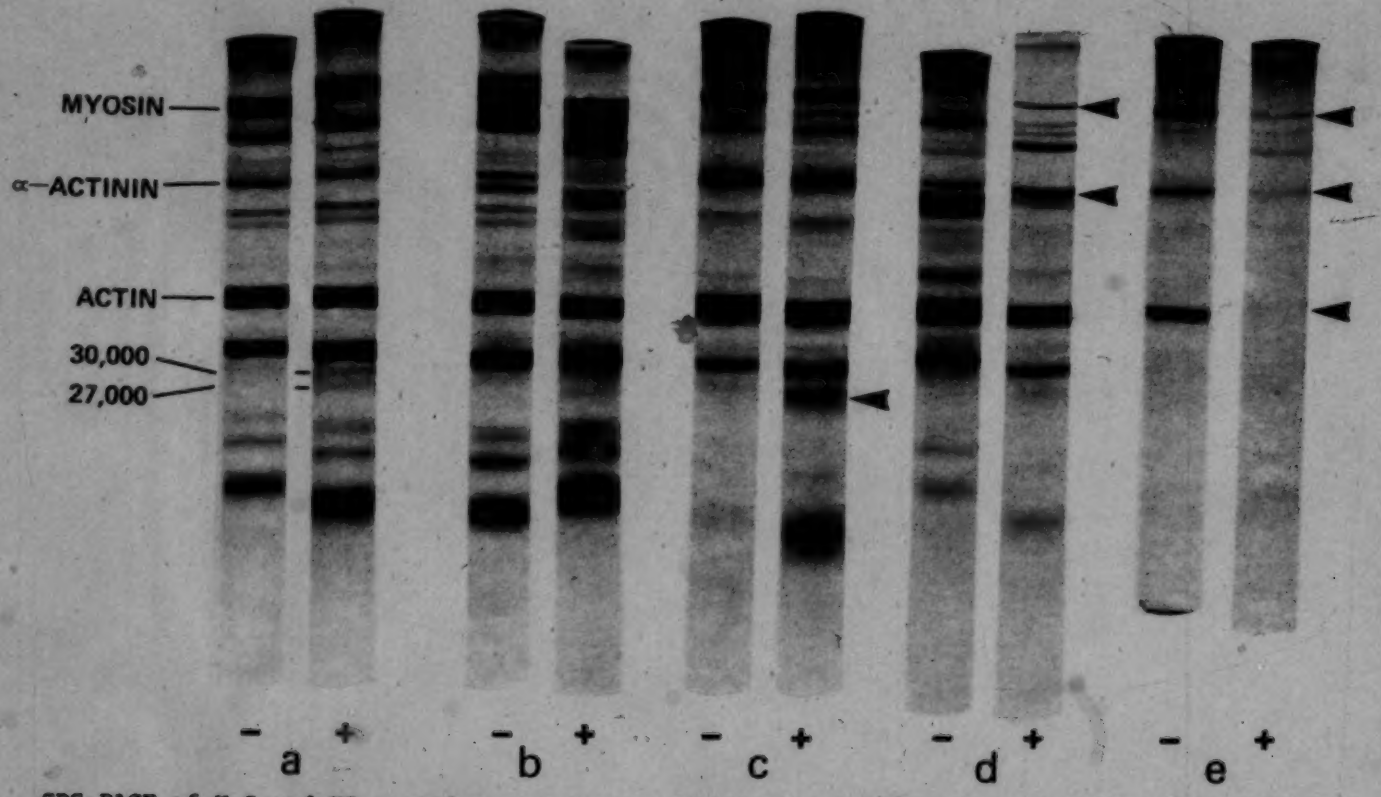

Plate 7. SDS-PAGE of H-S and KI protein extracts and insoluble residues after incubation with CAF. Myofibrils (25 mg/ml) were extracted with H-S and KI. Protein extracts or residues were incubated with CAF (0.7 units/ml) and 5 mM $CaCl_2$(+) or EDTA (-) for one hour at 25°C. (a) Myofibrils, 100 µg; (b) protein soluble in the first H-S extraction, 100 µg; (c) H-S insoluble residue, 100 µg; (d) protein soluble in the first KI extraction, 100 µg; (e) KI insoluble residue, 50 µg. Protein quantities represent those for the control (-) samples; equivalent volumes were applied to experimental (+) gels to facilitate direct comparison.

extensive aging and fourteen-hour CAF treatment of isolated intact myofibrils (see Plates 3b and 4).

KI soluble actin, in contrast, resists proteolysis by CAF. This control ensured that actin had not been altered during extraction in any way that might change the characteristics of its hydrolysis.

Furthermore, actin isolated by another method (Rees and Young, 1967), was not hydrolysed by CAF (Plate 8a). Nor did the state of polymerization affect the proteolysis of actin. G-actin prepared by the above method was polymerized to form F-actin. Plate 8b shows that F-actin was also not hydrolysed by CAF. Myofibril ghost actin included in Plate 8c for comparison was completely hydrolysed. The fact that actin is highly resistant to CAF hydrolysis has been well documented (Dayton et al., 1975; Ishiura et al., 1979). However, the actin that remained insoluble was easily hydrolysed. Since both actins had undergone identical KI treatment, it can be concluded that two proteins of similar MW existed in the myofibril, exhibiting differing solubilities in KI and with different susceptibility to proteolysis by CAF.

Characteristics of KI Insoluble "Ghosts"

H·S and KI extracted residues of myofibrils were prepared for study. Following KI extraction and resuspension in low ionic strength solution (0.15M NaCl, .03M Tris, 0.1mM EDTA), the ghosts became very sticky and adhered to glass (which complicated transfer) and formed clumps of highly insoluble flakes that were difficult to disperse. Ghosts resisted solubilization in a variety of solvents including 1M NaOH and 1M acetic acid, but were almost completely soluble in 8M urea

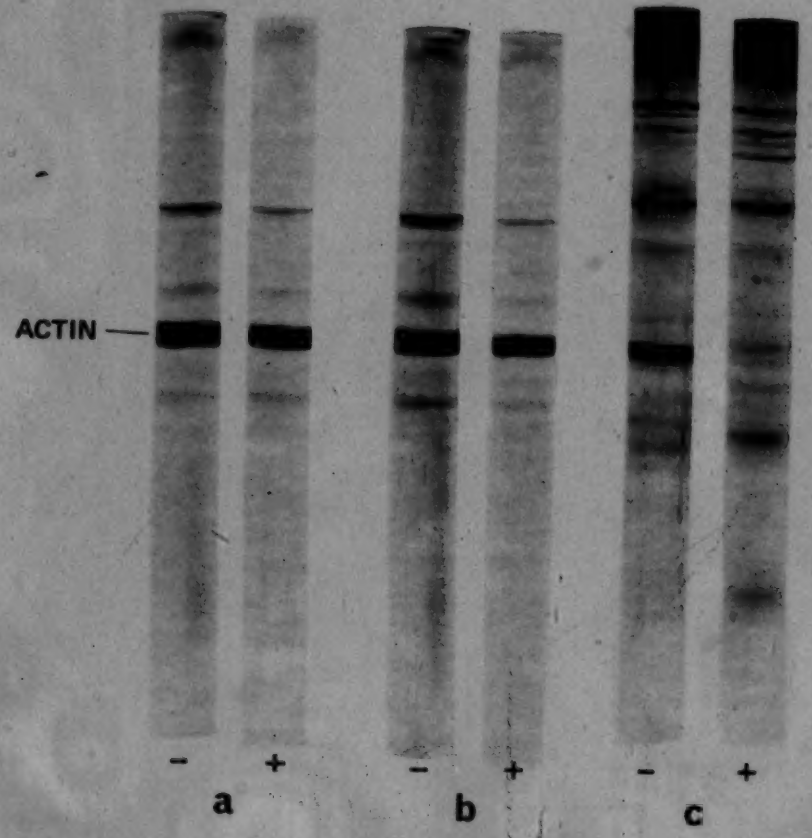

Plate 8. SDS-PAGE of purified G-actin and F-actin incubated with CAF: comparison of actin hydrolysis in KI insoluble ghosts and isolated actin samples. Purified actin (G-actin) and polymerized F-actin (both 1 mg/ml), and KI insoluble ghosts (6 mg/ml) were incubated with 0.7 units/ml CAF in the presence of 5 mM $CaCl_2$ (+) or EDTA (-) for one hour at 25°C. (a) G-actin, 20 µg; (b) F-actin, 20 µg; (c) KI insoluble ghosts, 40 µg. Protein quantities represent those for the control (-) samples; equivalent volumes were applied for the experimental (+) gels to facilitate comparison.

and one percent SDS containing 5mM 2-mercaptoethanol.

Plate 9 illustrates gels of two different preparations of ghosts (a) and (b). Both contain the proteins myosin, α-actinin and actin. However, in some preparations, two other bands of 123,000 and 85,000 daltons were observed (b).

Analysis of the solubility of the proteins of prepared ghosts yielded the following results. α-actinin could be easily solubilized using one percent SDS only (Plate 9c) but the solubilization of the remainder of the complex required the presence of 2-mercaptoethanol. It is likely that α-actinin is not associated by disulfide bridging with the rest of the complex, since it was easily extracted here with SDS. It was also easily dissociated from myofibrils in control incubations (see Plates 4 and 5b), and has been purified by low ionic strength extraction of myofibrils (Goll et al., 1972). In contrast, the actin and myosin require the inclusion of a disulfide reducing agent (2-mercaptoethanol or DTT) for solubility (d). These results indicate the possibility of disulfide bridges linking actin and myosin which may be responsible for the insolubility of the complex.

Following exhaustive extraction of ghosts by SDS-2-mercaptoethanol solution, a residue containing five percent of the original material on a dry weight basis remained stubbornly insoluble. This material might be an insoluble form of connective tissue.

Ca^{2+} Requirement for CAF Proteolysis

Several experiments using Ca^{2+} concentrations from 20 μM to 10 mM were conducted to determine if the Ca^{2+} requirement for the CAF

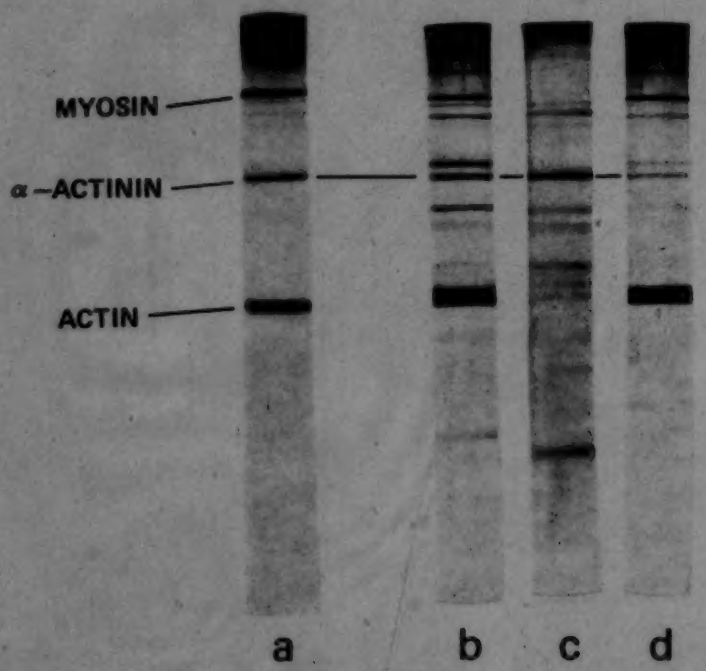

Plate 9. SDS-PAGE of KI insoluble ghost proteins extractable in SDS with and without 2-mercaptoethanol. (a) and (b) are gels of two different preparations of ghosts soluble in one percent SDS containing 5 mM 2-mercaptoethanol, 30 and 50 μg respectively; (c) ghost protein extracted by one percent SDS; (d) ghost protein insoluble in one percent SDS. The protein volumes applied in (c) and (d) were adjusted to reflect the proper relative amount of protein extracted from or remaining in ghost residues.

proteolysis of casein, myofibrils and ghosts was similar. These results are presented in Figure 5 . CAF attained a maximum rate of hydrolysis near 300 μM with all three substrates when Tris-acetate buffer was used.

The hydrolysis of actin in ghosts under the same conditions was confirmed in parallel reactions, again using Tris-acetate buffer, where the ghost proteins were applied to gels after incubation with CAF. These gels, shown in Plate 10, clearly illustrate that actin hydrolysis was complete at Ca^{2+} concentrations as low as 100 μM.

These results differ from those of Ishiura et al. (1978). Their relative enzymatic activity reached maximal activation only in the presence of 1.8 mM Ca^{2+} in the presence of sodium glycerophosphate buffer. By substituting their buffer into the reactions described above, optimum caseinolytic activity was achieved only with 1 mM Ca^{2+}. It was concluded that sodium glycerophosphate buffer inhibits CAF activity at low Ca^{2+} ion concentrations, most likely due to the formation of a soluble calcium glycerophosphate complex and thus effectively lowering the concentration of free Ca^{2+} ion. These results clearly show that Ca^{2+} activated CAF hydrolysis of the three substrates tested here occurs at much lower Ca^{2+} concentrations than previously determined for chicken CAF. The Ca^{2+} optimum for this enzyme differs also from that of porcine CAF which was determined to be 1 mM using Tris-acetate buffer (Dayton et al., 1975).

It was concluded that CAF recognized no differences between casein, myofibrils or ghosts with respect to Ca^{2+} ion activation. These results concur with those of Ishiura et al. (1978) who concluded that the role of Ca^{2+} was to activate the enzyme and had little or no effect on the sub-

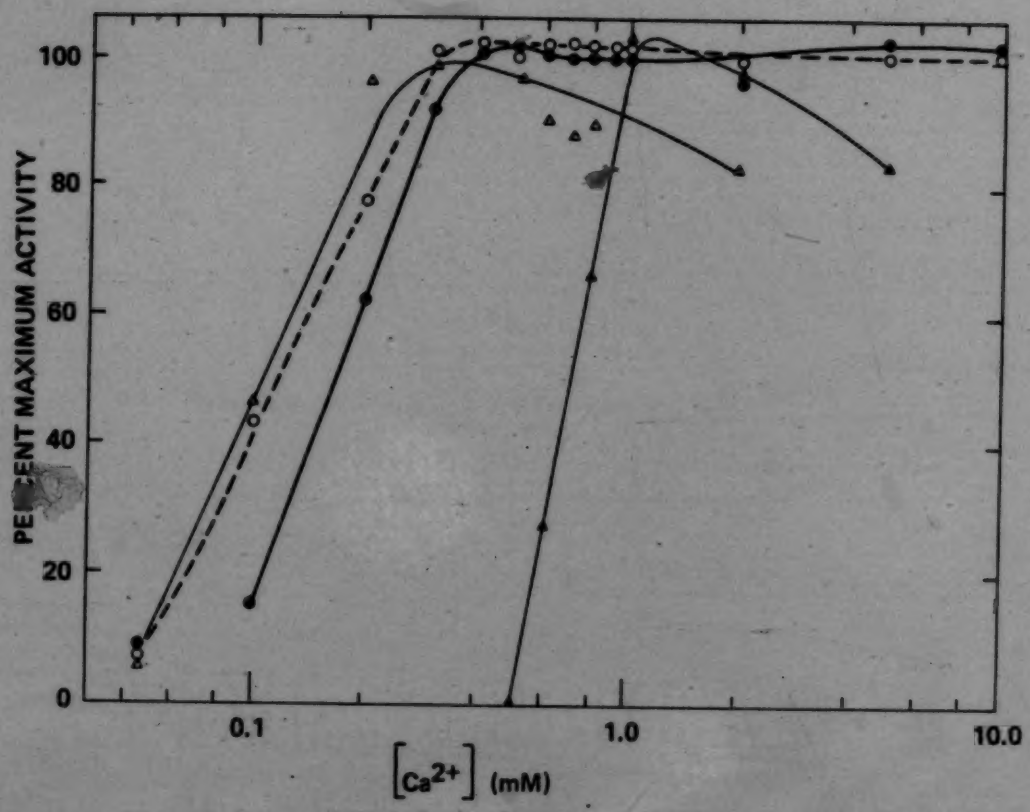

Figure 5. Effect of Ca^{2+} concentration on CAF activity. Standard reaction mixtures contained CAF (0.2 units/ml) and were adjusted to contain from 50 μM to 10 mM free Ca^{2+} ion. Substrates used were 0.24% casein (△——△), 12 mg/ml myofibrils (●——●) or 8 mg/ml ghosts (○– – –○). In one series of reactions, 0.1 M sodium glycerophosphate buffer pH 8.0 was substituted for the usual tris-acetate buffer and used with 0.24% casein substrate (▲——▲). Samples were incubated one hour at 25°C. In order to facilitate comparison of CAF activity between substrates, percent maximum activity was calculated relative to the maximum rate of hydrolysis observed for each substrate.

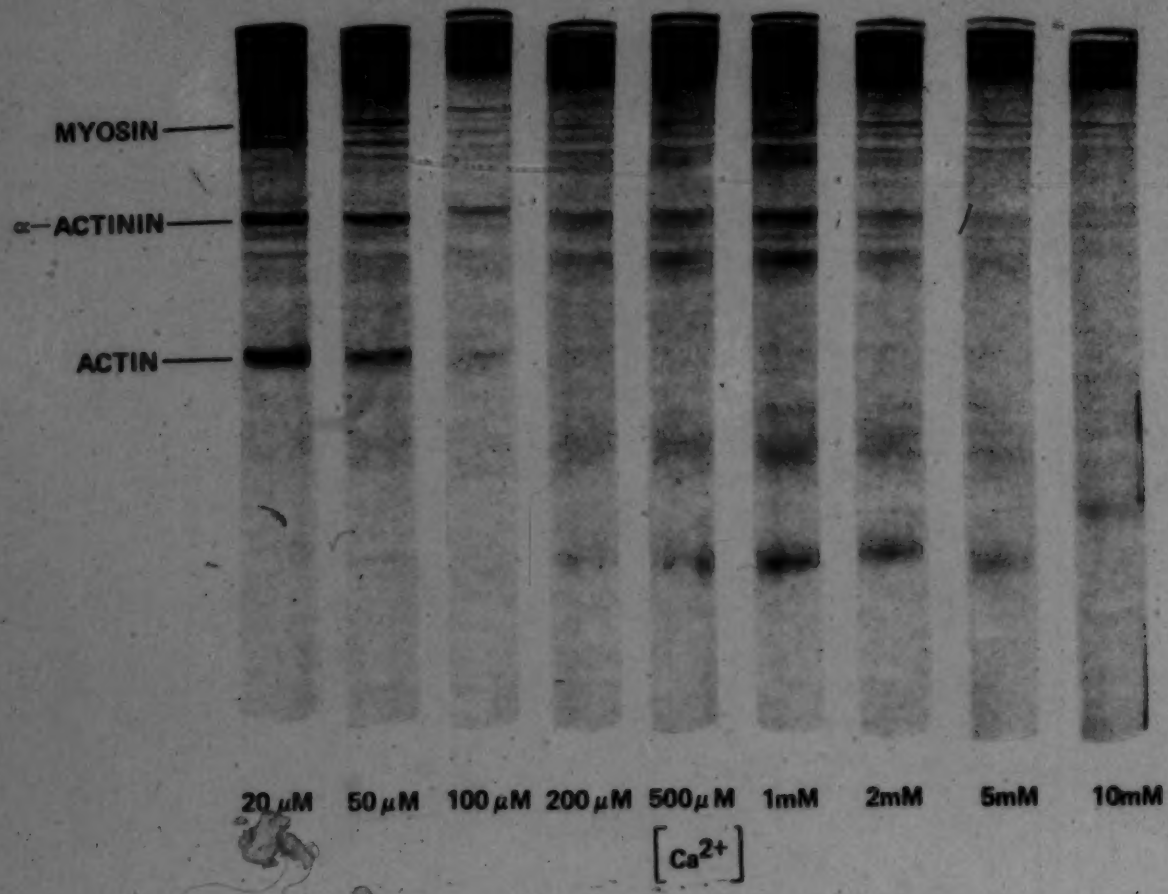

Plate 10. SDS-PAGE of myofibril ghosts incubated with CAF at increasing Ca^{2+} ion concentration. 6 mg/ml ghosts were incubated with CAF (0.2 units/ml final concentration) for one hour at 25°C in a standard reaction mixture. Free Ca^{2+} ion concentration was varied from 20 μM to 10 mM.

strate.

pH Optimum of CAF Proteolysis

To determine the pH profile of hydrolysis of ghost proteins, CAF activity on ghosts was measured at different pH's, and is presented in Figure 6. CAF activity was determined to be greatest at pH 7.8 but the profile was not symmetrical. Low levels of activity were still detectable at pH 5.5. Gels prepared from a parallel experiment indicate that hydrolysis of actin occurred over a wide range of pH (Plate 11), and was detectable even at pH 5.0. These results are in good agreement with the pH optimum of 7.7 found by Ishiura et al. (1978) using casein as a substrate.

Thus, while the pH optimum of CAF lies near pH 7.8, the hydrolysis of ghost actin was still detectable at and below the pH of post-mortem muscle (pH 5.4 to 6.0).

Isolation of Pure Ghost Actin

Actin in ghosts differed substantially from KI soluble actin in both solubility in KI and in susceptibility to CAF hydrolysis. In order to determine if further differences in the two molecules existed that might explain these observations, the actins first had to be purified.

Several problems arose in perfecting a feasible procedure for actin isolation. First, it was necessary to isolate the proteins by the same method to ensure that artifacts were not generated through the use of different isolation procedures. This presented special problems for the ghost actin because of its insolubility.

Separation using molecular filtration chromatography on AcA 22 (LKB, France) in 1% SDS and 5mM DTT was only partially successful. Ghost

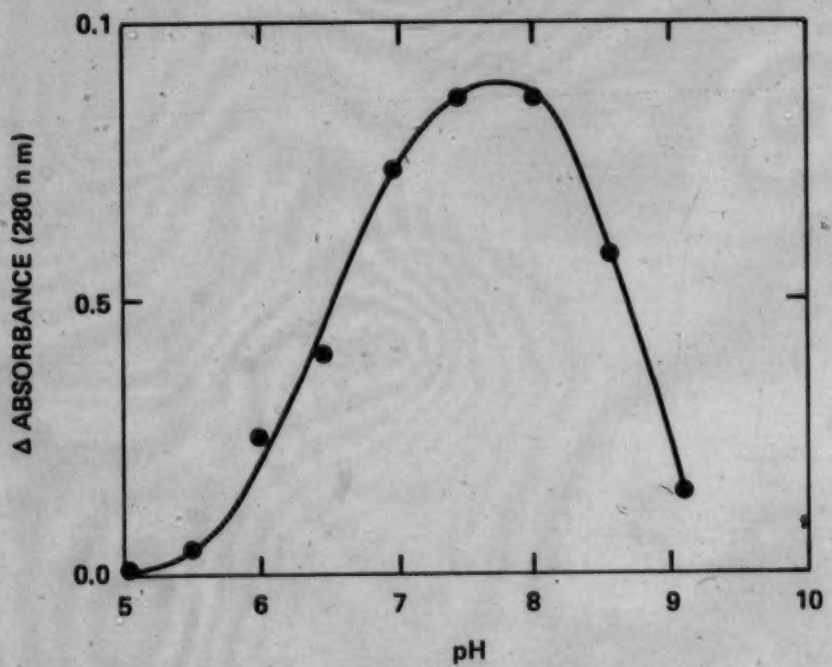

Figure 6. Effect of pH on CAF activity using a KI insoluble ghost preparation as a substrate. The standard incubation mixture included 6 mg/ml ghosts and 0.2 units/ml CAF activity. The pH of reaction mixtures was adjusted to between pH 5 and pH 9 at approximately 0.5 unit intervals. Absorbance at 280 nm was read after 60 min. against an EDTA blank.

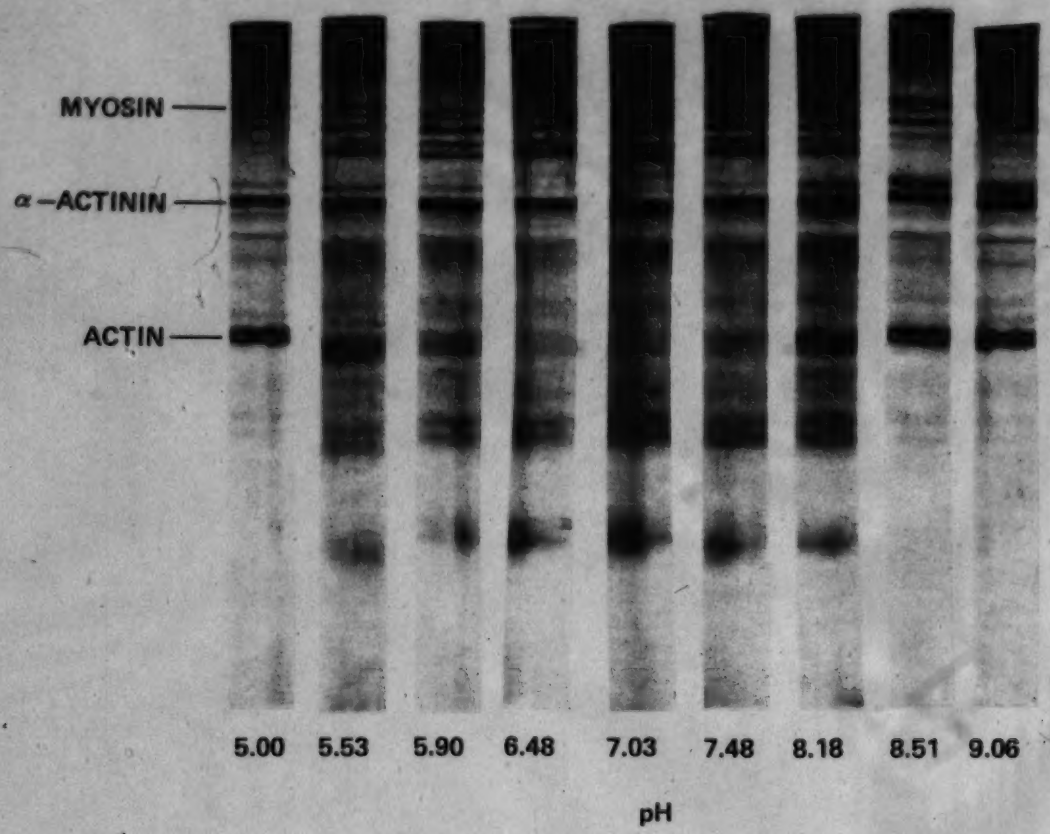

Plate 11. SDS-PAGE of myofibril ghosts following incubation with CAF at increasing pH values. Conditions of incubation were identical to those described in Figure 6. 65 µg protein was applied to each gel.

actin was soluble and was separated from α-actinin and myosin but the inherent limitations in resolution of gel filtration precluded complete separation of actin (44,000 daltons) from desmin (50,000 daltons), the protein subunit of intermediate filaments, also insoluble in KI (Granger and Lazarides, 1978). It was essential that desmin not be present in the purified actin preparation, since the actin was to be used in amino acid analysis and in the production of antibodies described later in this work.

The same problems with separation of actin and desmin were encountered using conventional preparative SDS gel electrophoresis. While separation by electrophoresis was good, the mixing encountered in eluting the protein from the gel column produced similar resolution to that accomplished by gel filtration.

The problems described here were also encountered in the purification of desmin from chicken gizzard by Hubbard Lazarides in 1979. Desmin was persistently accompanied by actin during isolation. They finally succeeded in isolating a fraction of the desmin by gel filtration in 1 M acetic acid, but actin was never purified. A technique described earlier (Lazarides, 1976) was adapted for use in this work, and several milligrams of actin were purified from ghosts and KI soluble material using SDS-gels. Each individual gel was scanned at 280 nm and the bands corresponding to actin were cut out and collected. Protein was eluted electrophoretically and SDS removed by passage through an ion exchange column.

KI soluble protein was easily prepared by this method. However, loading of large quantities of ghost protein onto these gels had to be avoided because of gel shrinkage and band distortion. Thus, only 100 µg of ghost protein could be applied to each gel. The purity of actins isolated by this procedure can be seen in Plate 12. No desmin was

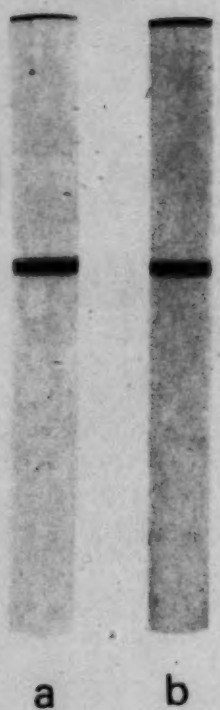

Plate 12. SDS-PAGE of KI soluble and ghost actin isolated by preparative gel electrophoresis. (a) KI soluble actin, 20 µg ; (b) actin from ghosts, 20 µg.

evident in either preparation. The $A^{1\%}_{1cm}$ at 280 nm (the absorbance for a one percent solution in a one centimetre cuvette) value for both proteins was determined. The values for KI soluble actin (15.5) and ghost actin (16.0) were nearly identical for the SDS- purified actins but differed from that determined for undenatured actin 10.9 (Rees and Young, 1967). The difference was probably due to the state of denaturation of the proteins following purification.

Hydrolysis of Isolated Actins by CAF

The results of an experiment comparing the hydrolysis of KI soluble and ghost actin (both isolated by preparative SDS gel electrophoresis) are shown in Plates 13c and 13d. The hydrolysis of intact myofibrils (a) and ghosts (b) is included for reference. The pattern of degradation products in both actins was identical. After twenty minutes' digestion, major degradation products of 26,000 and 18,000 daltons, and also a minor 24,000 dalton band were observed. In control gels containing only CAF and no substrates, no such degradation products were produced (e). Therefore, all new bands arose from proteolysis of the substrates. Hydrolysis of actin in intact ghosts showed the same degradation products, but hydrolysis occurred more rapidly than in the isolated actins. In myofibrils, the 27,000 dalton product of TN-T hydrolysis appeared at the same time that the 26,000 dalton product appeared in gels of the intact ghost degradation and in both cases, by 240 minutes, both products had decreased slightly in intensity. Thus the possibility exists that the 27,000 dalton band observed in gels of myofibril degradation may result partly from the degradation of ghost actin hydrolysis. In some gels (see Plates 1 and 4), the 27,000 dalton band was much more

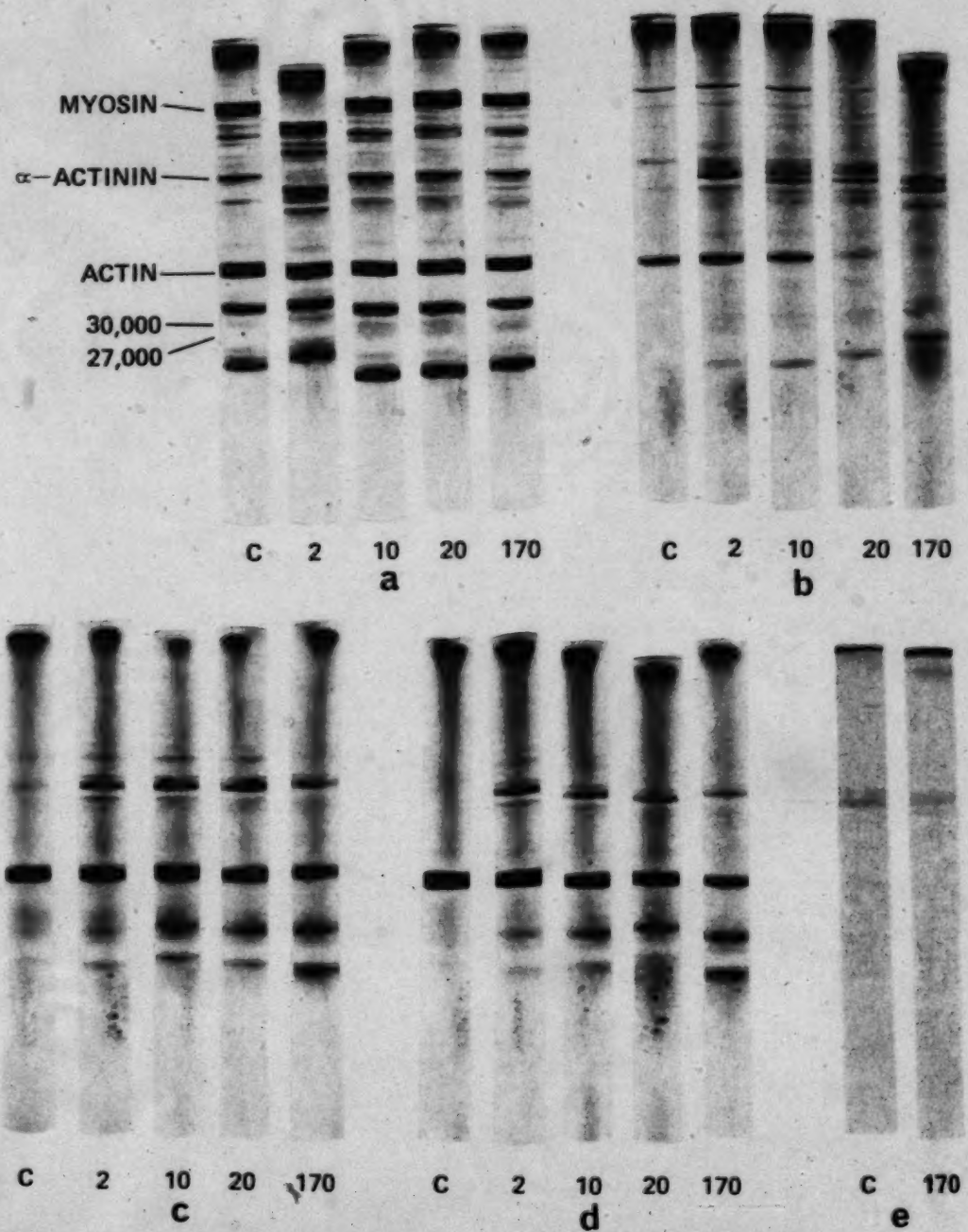

Plate 13. SDS-PAGE of myofibrils, ghosts, and isolated actins: time course of hydrolysis by CAF. Standard reaction mixtures contained CAF (0.2 units/ml) and the following substrates: (a) 12 mg/ml myofibrils, 100 µg/gel; (b) 6 mg/ml ghosts, 30 µg/gel; (c) 200 µg/gel KI soluble actin, 20 µg/gel; (d) 200 µg/ml ghost actin, 20 µg/gel and (e) CAF only in the same concentrations as in (a) to (d). Actins in both (c) and (d) were isolated by preparative gel electrophoresis. Reactions were incubated at 25°C and aliquots were removed prior to CAF addition (C) and at 2, 10, 20 and 170 min. after CAF addition.

intense than the 30,000 dalton band, and the development of the two bands did not always coincide. However, in published studies of purified TN-T hydrolysis in pork (Dayton et al., 1975) and chicken (Ishiura et al., 1979), gels have shown products of equal intensity. The results presented here show that following isolation, both KI soluble and ghost actin had identical degradation products, and were degraded at the same rate. The fact that both actins underwent a slower hydrolysis in comparison with ghost hydrolysis indicated that some alteration had occurred during the isolation procedure. Since modification of the primary structure of the molecules was unlikely, the results indicate that conformation or some other subtle and yet undetermined property may be responsible for the observed susceptibility to proteolysis of actin in myofibril ghosts.

CAF Hydrolysis of Denatured Myofibril Proteins

To determine if the hydrolysis of the actin in myofibrils might be affected by its conformation, isolated myofibrils were subjected to 0.1 M HCl, 0.1 M NaOH, or 8 M urea for three hours or to 100°C heat for five minutes to denature the constituent proteins, neutralized where necessary, dialysed, then incubated with CAF. In all experiments, gels showed that proteolysis was identical. Plate 14 shows gels representative of all experiments. In each case, CAF hydrolysed myosin, α-actinin, actin and tropomysin producing low molecular weight degradation products (a). After undergoing the same denaturation, KI soluble actin was also hydrolysed (b).

The degradation of denatured actin illustrates that the conformation of actin affects its proteolysis by CAF. The hydrolysis previously observed in KI denatured myosin and α-actinin also indicated that denaturation was instrumental in their susceptibility to proteolysis by

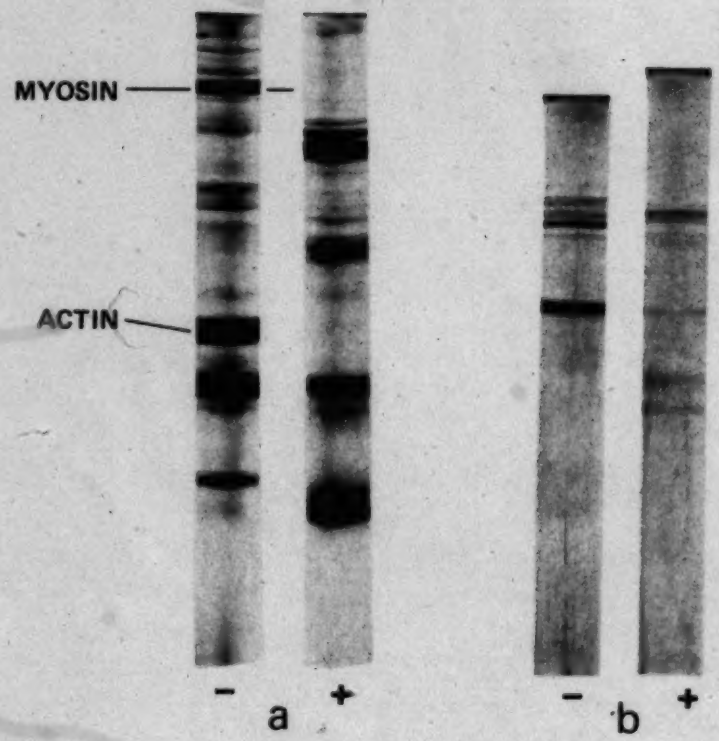

Plate 14. SDS-PAGE of myofibrils and KI soluble myofibril protein denatured with NaOH and incubated with CAF. Myofibril protein (12 mg/ml) and KI soluble protein (4 mg/ml) were denatured with NaOH as described in materials and methods, then incubated with CAF (0.2 units/ml) and 5 mM $CaCl_2$ (+) or EDTA (-) at 25°C for 60 min. (a) myofibrils, 100 µg; (b) KI soluble protein, 25 µg.

CAF. These results are supported by the observations of Ishiura et al. (1979) who discovered that CAF digests most proteins once they are denatured.

Isoelectric Focusing of Isolated Actins

Isoelectric focusing was used to determine if a difference existed in the isoelectric points of KI soluble and ghost actin. The different isoelectric point might reflect differences in amino acid composition which could be responsible for differences in conformation between actins.

IEF gels of the individual actins show single bands (Plates 15a and 15b). Combined, the proteins focus in different regions of the pH gradient within the gel (c). The resolution of the combined proteins is a function of different isoelectric points unique to actin from each source. Since the isoelectric pH occurs where the net charge of the constituent amino acids is equal to zero, different isoelectric pH's reflect unique amino acid compositions.

CAF Hydrolysis of Smooth Muscle Protein

Three isoelectric variants α, β and γ actin have been observed in chicken muscle. It has been previously shown (Izant and Lazarides, 1977) that α-actin is the major myofibril actin component, and is preferentially extracted with KI. γ-actin has been shown to be insoluble in KI. γ-actin has also been shown to be the predominant form of actin in chicken smooth muscle (gizzard) contractile proteins. Incubation of CAF with contractile proteins of smooth muscle (Plate 16) and the KI extracted residue of smooth muscle (b) demonstrated that a partial hydrolysis of actin occurred

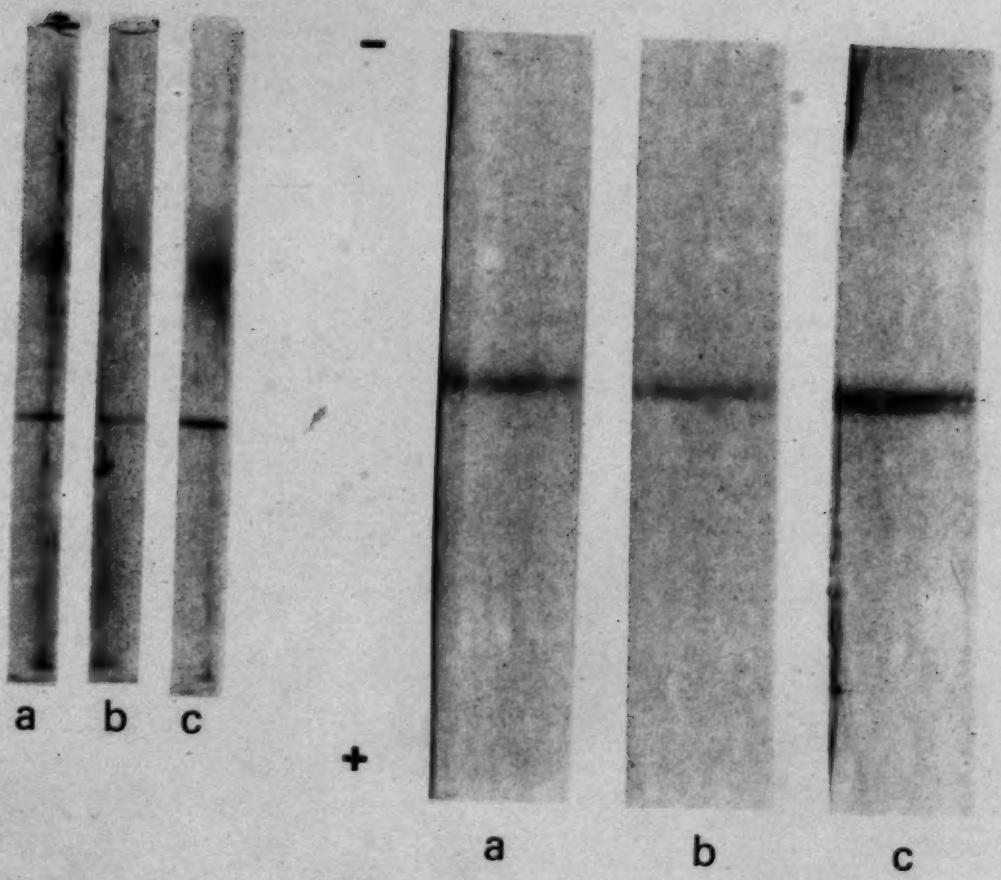

Plate 15. Isoelectric focusing of actins isolated by preparative gel electrophoresis. (a) KI soluble actin, 10 µg; (b) ghost actin, 10 µg and (c) both KI soluble and ghost actins combined, 10 µg each. Magnified sections of the same gels are also illustrated.

Plate 16. SDS-PAGE of smooth muscle protein incubated with CAF. 2 mg/ml smooth muscle protein prepared by sedimentation of homogenates of chicken gizzard tissue (a) and 1 mg/ml of the same protein extracted with KI (b) were incubated with CAF (0.2 units/ml) in the presence of 5 mM $CaCl_2$(+) or EDTA (-) for 60 min. at 25°C. 20 µg smooth muscle protein (a) and 40 µg KI extracted protein were applied to each gel.

in intact contractile proteins. Only forty-seven percent of the actin remained (determined by densitometry). Complete hydrolysis occurred in the KI residues. These studies imply that CAF hydrolyses the γ-variant of actin found in insoluble residues of KI extracted myofibrils.

Amino Acid Analysis of Isolated Actins

The amino acid analysis of the isolated actins is presented in Table 3 and compared with data derived by sequencing rabbit actin (Kuehl et al., 1975), claimed identical to chicken muscle actin. The results show that the amino acid analysis of actins from the two sources are indistinguishable. This analysis confirms that the KI insoluble protein in Z-discs is nearly identical with KI-soluble actin. However, minor differences in one or more amino acids that might explain the difference in isoelectric mobility cannot be conclusively determined from amino acid data.

The results are in good agreement with published values which were determined by sequencing and are therefore highly accurate. The low value determined for threonine may be due to its hydrolysis during acid digestion. Cysteine was reduced to almost undetectable levels under the conditions of hydrolysis. The four residues of tryptophan are also undetected, as tryptophan normally undergoes complete hydrolysis under these conditions and cannot be determined by this procedure. Incomplete hydrolysis of the peptide bonds involving valine and isoleucine is often observed and may be responsible for low values for these two amino acids. The low values for tyrosine are unexplained.

The results presented here show that both KI soluble and ghost actin have very similar amino acid compositions. This eliminates the possibility that the protein in ghosts is a completely different protein than actin but has a similar molecular weight.

TABLE 3

Amino Acid Analysis of SDS-PAGE Purified KI Soluble and Ghost Actins

Amino Acid	Number of Residues (Mols/Mol) Established by Sequence*	Determined in This Study: KI Soluble Actin	Determined in This Study: Ghost Actin
Lys	19	19.2	19.7
His	8	8.6	7.9
His (τ Me)	1	---	---
Arg	18	15.9	17.0
Asx	34	30.1	30.9
Thr	27	21.6	21.5
Ser	22	21.3	22.4
Glx	39	39.6	40.4
Pro	19	19.7	21.1
Gly	28	33.8	31.2
Ala	29	28.9	29.8
Cys	5	~0	~0
Val	21	16.3	17.5
Met	16	12.7	12.0
Ile	30	22.2	21.3
Leu	26	24.1	25.4
Tyr	16	12.2	10.6
Phe	12	10.4	10.6
Trp	4	---	---

* From Kuehl et al. (1975)

CAF Proteolysis of Bovine Ghost Actin

In order to determine that the actin hydrolysis observed was not specific to only the chicken system, similar experiments were repeated using isolated beef myofibrils and a crude CAF preparation also from beef muscle. Since similar degradative changes occur in the beef system (Olson et al., 1977), the hydrolysis of ghost actin in beef was likely.

An isoelectric fraction of the soluble bovine sarcoplasmic protein that contained CAF activity was incubated with isolated beef myofibrils. It was established that the CAF preparation possessed a Ca^{2+} requirement of 1 mM (Figure 7) and required a pH of 8.0 (Figure 8) for optimum activity, similar to that for isolated chicken CAF. No proteolysis was detectable in this preparation at pH 8.0 in the absence of free Ca^{2+}. Plate 17a shows that CAF produces changes in the beef system which are similar to those observed in chicken myofibrils. The α-actinin band decreased in intensity and 30,000 and 27,000 dalton bands appear. KI insoluble ghosts of beef myofibrils had the same composition as chicken ghosts. These contained myosin, α-actinin and actin. CAF treatment shown in Plate 17b demonstrates that ghost actin is easily hydrolysed. Purified actin by the method of Rees and Young (1967) was not hydrolysed (c).

The results presented here point out that the proteolysis of ghost actin observed in chicken is not restricted to just that species but occurs in beef and likely occurs in pork, rabbit and other systems where similar post-mortem degradation has been detected.

Production of Anti-Actin Antiserum

Antibody production in mice was undertaken using the preparative

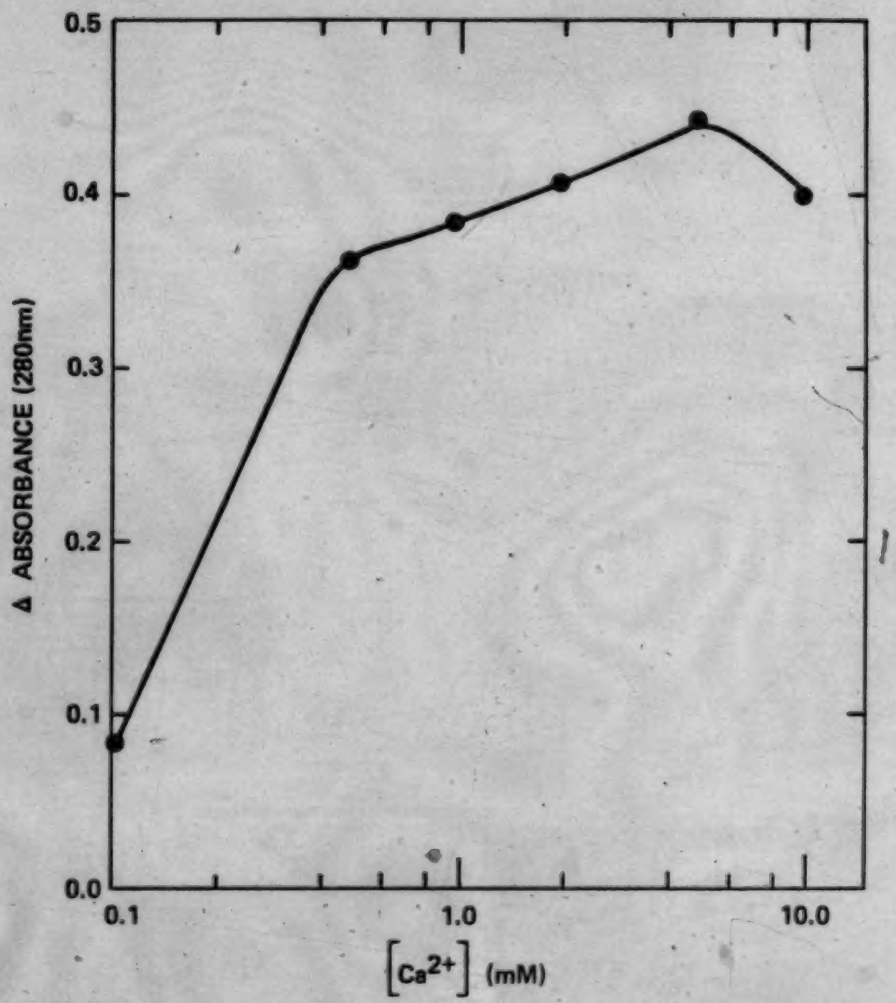

Figure 7. Effect of Ca^{2+} ion concentration on the activity of beef CAF on beef myofibrils. A standard incubation mixture included 15 mg/ml myofibrils from 100 μM to 10 mM free Ca^{2+} ion and a crude CAF preparation (0.6 units/ml) was incubated at 25°C. The absorbance at 280 nm of the supernatant solution was read after 30 min. against a blank containing CAF and 5 mM EDTA.

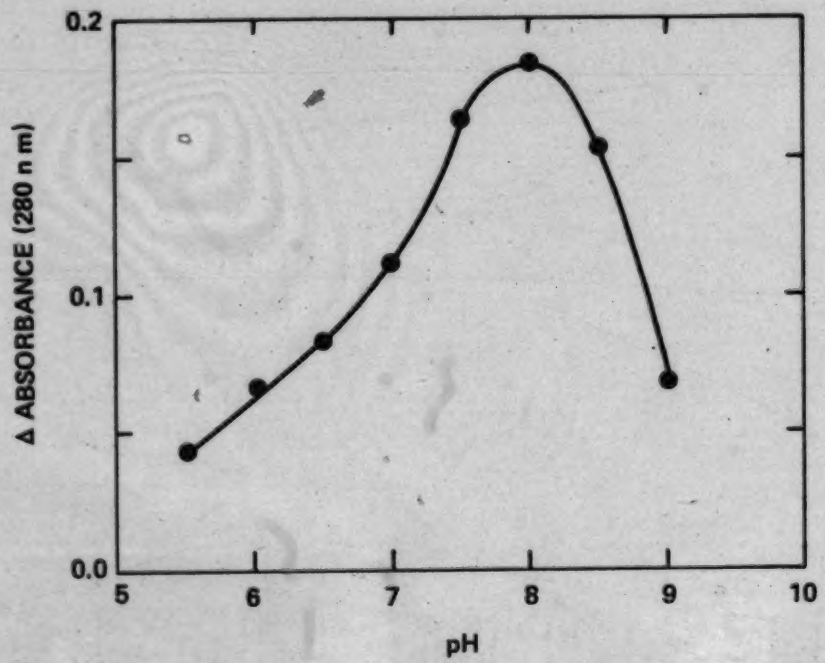

Figure 8. Effect of pH on the activity of beef CAF on beef myofibrils. Standard incubation mixtures containing 15 mg/ml myofibrils, and a crude CAF preparation containing 0.6 units CAF activity was incubated at 25°C. pH of reaction mixtures was adjusted to values between pH 5.0 and 9.0 prior to CAF addition. Reactions were stopped after 20 min. and the change in absorbance of the supernatant solution was read against a blank containing CAF and 5 mM EDTA.

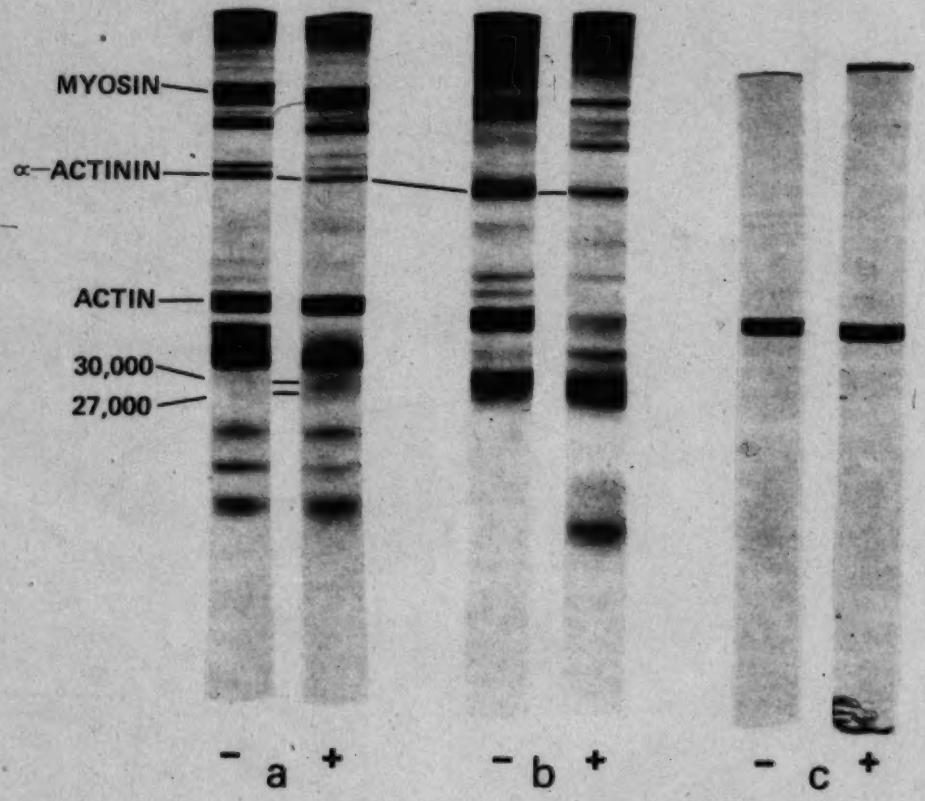

Plate 17. SDS-PAGE of beef myofibrils, H-S and KI extracted myofibrils and isolated actin incubated with beef CAF. Standard reactions contained 15 mg/ml myofibrils, 8 mg/ml H-S and KI extracted myofibrils or 200 µg/ml isolated beef actin, and also contained a crude CAF fraction containing 0.6 units/ml activity and 5 mM $CaCl_2$ (+) or EDTA (-). Incubation was at 25°C for one hour. Gels depict (a) myofibrils, 100 µg; (b) KI insoluble residue, 80 µg; (c) purified actin, 20 µg.

gel-purified KI soluble actin and ghost actin as antigens. The rationale for this approach was based on the possibility that the two molecules may be immunologically distinguishable. Historically, anti-actin antibodies are difficult to raise presumably because few species-specific differences exist; the structure of actin is highly conserved. Recently, however, antibody production to SDS-denatured actin has been reported (Lazarides and Weber, 1974).

Antibodies were detectable after the third immunization with ghost actin. In contrast, even after the fifth round of immunization using KI soluble actin, no antibody was detectable with the double immunodiffusion technique (Ouchterlony, 1968). The precipitin reaction between anti-ghost actin serum and both KI soluble and ghost actins is shown in Plate 18. The complete absence of spur formation at the point of contact of the precipitin lines establishes that both actins are antigenically identical.

It is not entirely unexpected that antibodies are not raised by direct immunization with KI-soluble actin especially if it is very similar to the mouse's own actin. However, once antibodies are raised to a nearly identical molecule (ghost actin), these antibodies completely cross-react with KI-soluble actin. This indicates immunologic identity at the B-cell level. Indeed, this may reflect certain differences between the actins, recognized by the T helper cells rather than the antibody-producing B cells of the immune system (Miller and Mitchell, 1968).

Localization of Actin in the Z-Disc by Indirect Immunofluorescence

Using fractionated anti-ghost actin antiserum, indirect immuno-

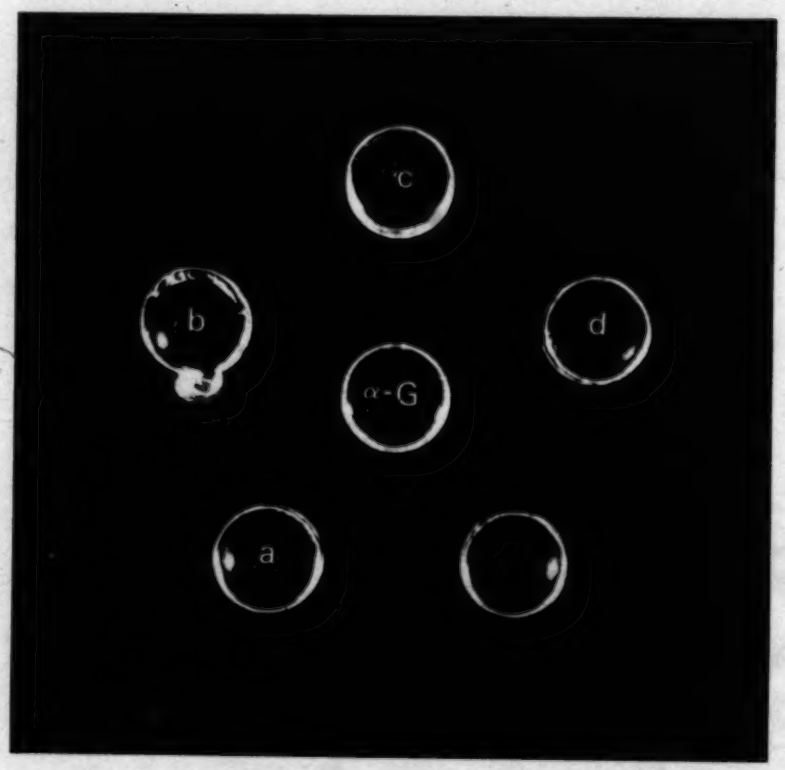

Plate 18. Double-immunodiffusion of fractionated anti-ghost actin serum against isolated KI soluble and ghost actin. Ammonium sulfate fractionated serum produced against ghost actin (α-G, center well) was challenged with SDS-PAGE-purified KI soluble and ghost actin. Ghost actin: (a) 800 µg/ml and (c) 400 µg/ml. KI soluble actin (b) 800 µg/ml and (d) 400 µg/ml.

fluorescence was used to determine the exact location of actin in glycerinated myofibrils. Homogenized muscle fibers adhering to coverslips were incubated first with the anti-ghost actin antiserum, then with commercially available FITC-conjugated anti-mouse IgG $F(ab')_2$ fraction.

Phase contrast and fluorescence microscope photographs of individual myofibrils or myofibril bundles are presented in Plate 19. In myofibrils (B), prominent fluorescence was observed primarily at the Z-discs of myofibrils with weaker fluorescence observed at the M-line (which did not appear clearly in all photographs). General fluorescent staining occurred across the entire myofibril. A very weak non-specific fluorescence background occurred in control myofibril preparations treated with normal (non-immune) mouse serum followed by the FITC fraction (A). No fluorescence occurred when preparations were treated with the FITC fraction alone (photographs not shown). In addition, no autofluorescence was detectable.

The results indicate that a higher concentration of actin exists in the Z-disc than in the rest of the sarcomere, based on the reaction identity between the two actins in the immunodiffusion studies.

When KI-soluble actin had been extracted from the myofibrils, only fluorescence at the Z-disc was observed (D). This result establishes that KI insoluble actin exists only at the Z-discs in extracted myofibrils.

Myofibrils on coverslips were incubated overnight with solutions of CAF containing EDTA or Ca^{2+}. Immunofluorescent labelling of the actin at the Z-disc seen in the EDTA control (E) was completely absent. Inspection of both phase contrast photographs revealed open gaps between I zones where the Z-disc material had once existed. Actin fluorescence

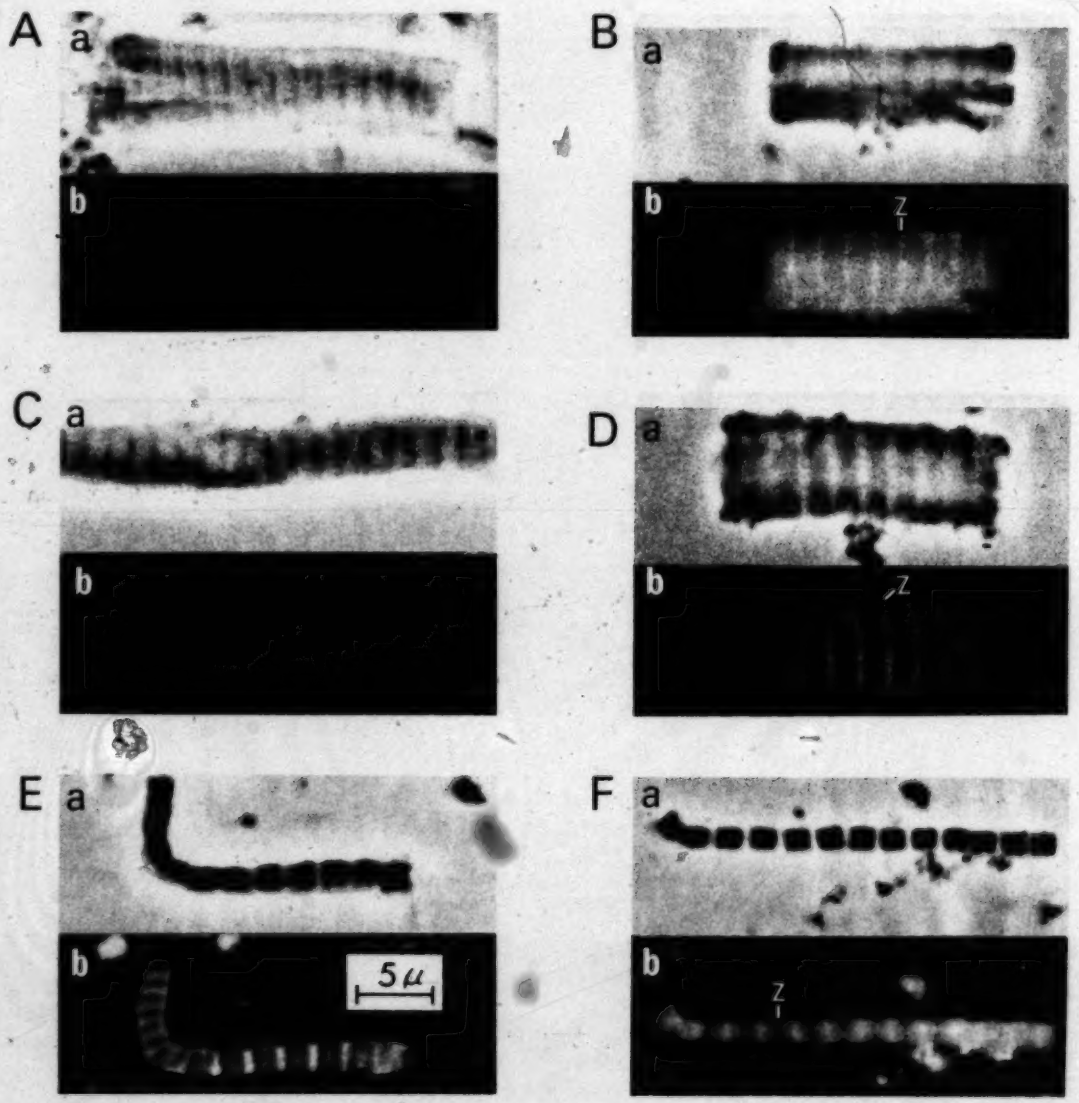

Plate 19. Indirect immunofluorescent labelling of ghost actin in myofibrils, KI extracted myofibrils and myofibrils incubated with CAF. Paired phase contrast (a) and fluorescence (b) micrographs of FITC labelled anti-ghost actin (α-G) treated glycerinated muscle myofibrils and extracts. A. Myofibrils - non-immune serum control. B. Myofibrils - α-G serum treated. C. KI-extracted myofibrils - non-immune serum control. D. α-G serum treated. E. Myofibrils incubated with CAF - EDTA control. F. Myofibrils incubated with CAF and Ca^{2+}.

in the residual sarcomere remained as previously described. These results show conclusively that actin located at the Z-disc is removed by proteolysis by CAF. Thin filament actin remains unaltered.

ELECTRON MICROSCOPY

SDS-PAGE has been used in the examination of the protein components of control and CAF-treated myofibrils extracted using H·S and KI (Plates 4 and 5). Electron microscopy of similarly treated and extracted muscle fibers was performed to compare the results of the gel data with observed sarcomere ultrastructure.

Individual glycerinated fiber bundles were incubated with CAF + Ca^{2+} or control solutions containing either CAF + EDTA or only Ca^{2+} for ninety minutes. The conditions of incubation were similar to that for the one-hour incubation with CAF presented in Plate 5 except that the time of incubation was extended to ninety minutes to allow for the penetration of the enzyme. No differences between the Ca^{2+} only and CAF + EDTA controls were detected. Therefore only the EMs of CAF + EDTA are presented here as controls.

Entire Muscle Fibers

Muscle white fibers are characterized by large diameter myofibrils (65-70 μ), few mitochondria, narrow Z-discs and a poorly developed sarcoplasmic reticulum in the region of the H-Zone, in comparison to intermediate or red fibers. The muscle sections in Plate 20 were typical of white fibers. The darker staining A-band, central in the sarcomeres, consists of interdigitating thick and thin filaments. Individual thick filaments

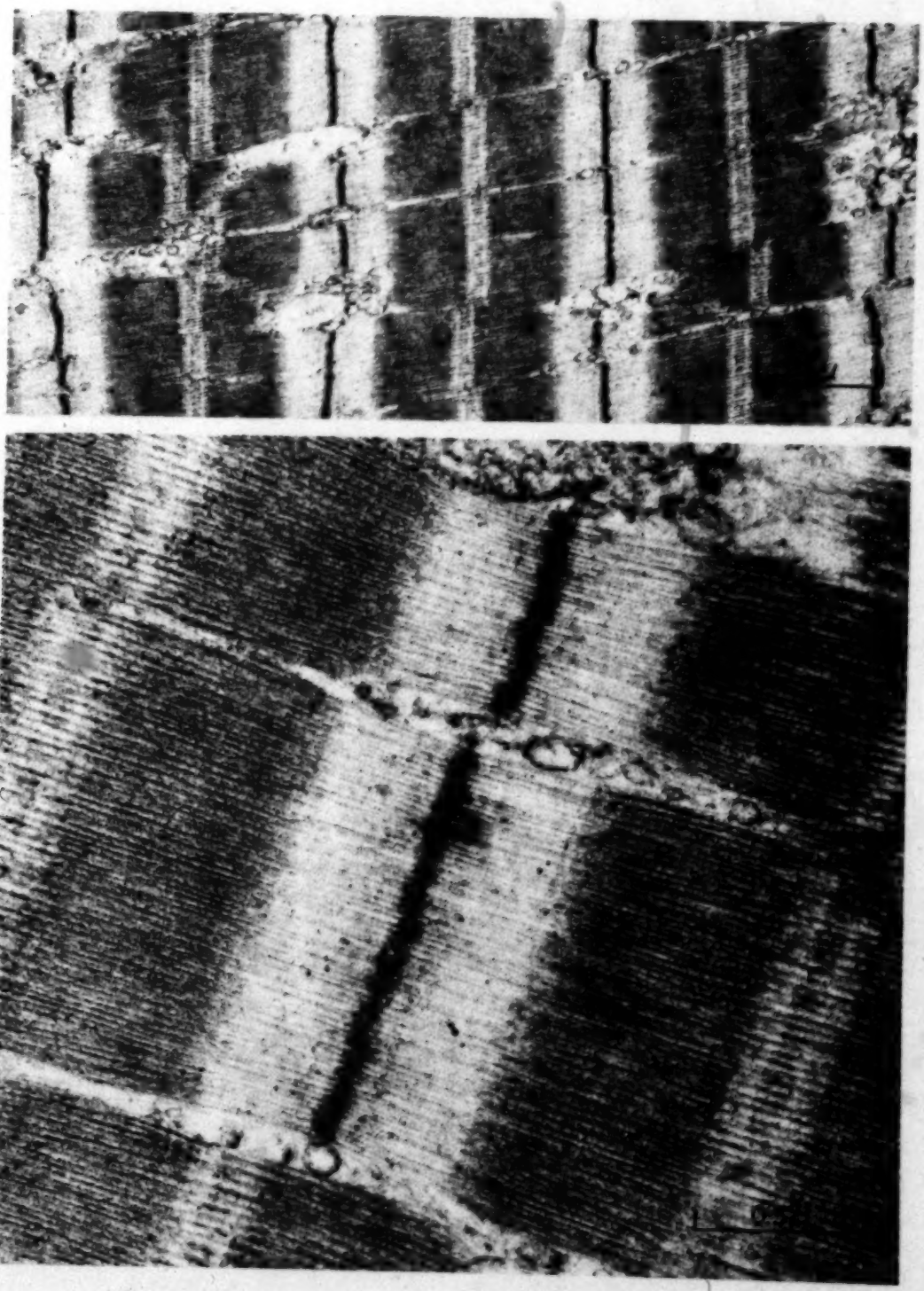

Plate 20. Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM EDTA.

were easily observed. The H-band at the centre of the A-band due to the absence of thin filaments was not easily visible. The pseudo-H-Zone due to the bare central region of the thick filament was easily seen. M-lines bisecting the pseudo-H-Zone appeared as a thickening of the thick filament at higher magnification. Some connections between the thick filaments could be seen at the M-line. In some EMs, weak bands with 41 nm periodicity, perhaps due to C-protein, were observed at the outer edges of the A-bands.

In the I-band, the individual thin filaments were visible. The thin filaments became thicker at the I-Z junction. In the Z-disc, a double row of branching thin filaments could be seen. The entire Z-disc structure was ~ 70 nm wide, typical of Z-discs in white fibers.

The T-system and sarcoplasmic reticulum (SR) were visible between myofibrils, located primarily near the I band. A few small mitochondria were seen near the Z-discs. Since glycerol extraction of muscles improves the contrast of the myofibril filaments but damages the membraneous organelles, the mitochondria, SR and T-system are indistinct.

Following incubation with CAF + Ca^{2+} the most prominent modification in myofibril structure was the complete removal of Z-disc structure (Plate 21). Wavy thin filaments remained in the I band separated by a gap where the Z-disc once existed. Sarcomeres of adjacent myofibrils were dramatically out of register in many samples, an indication that the intermediate filaments connecting the Z-discs may also have been affected. The T-system and SR appeared scattered and in many sarcomeres appeared within the I band. Their disarray may also be due to the effects of CAF on the intermediate filaments.

Many of the remaining sarcomeres were diagonally skewed. In general, the spacing and arrangement of the thick filaments in A-bands

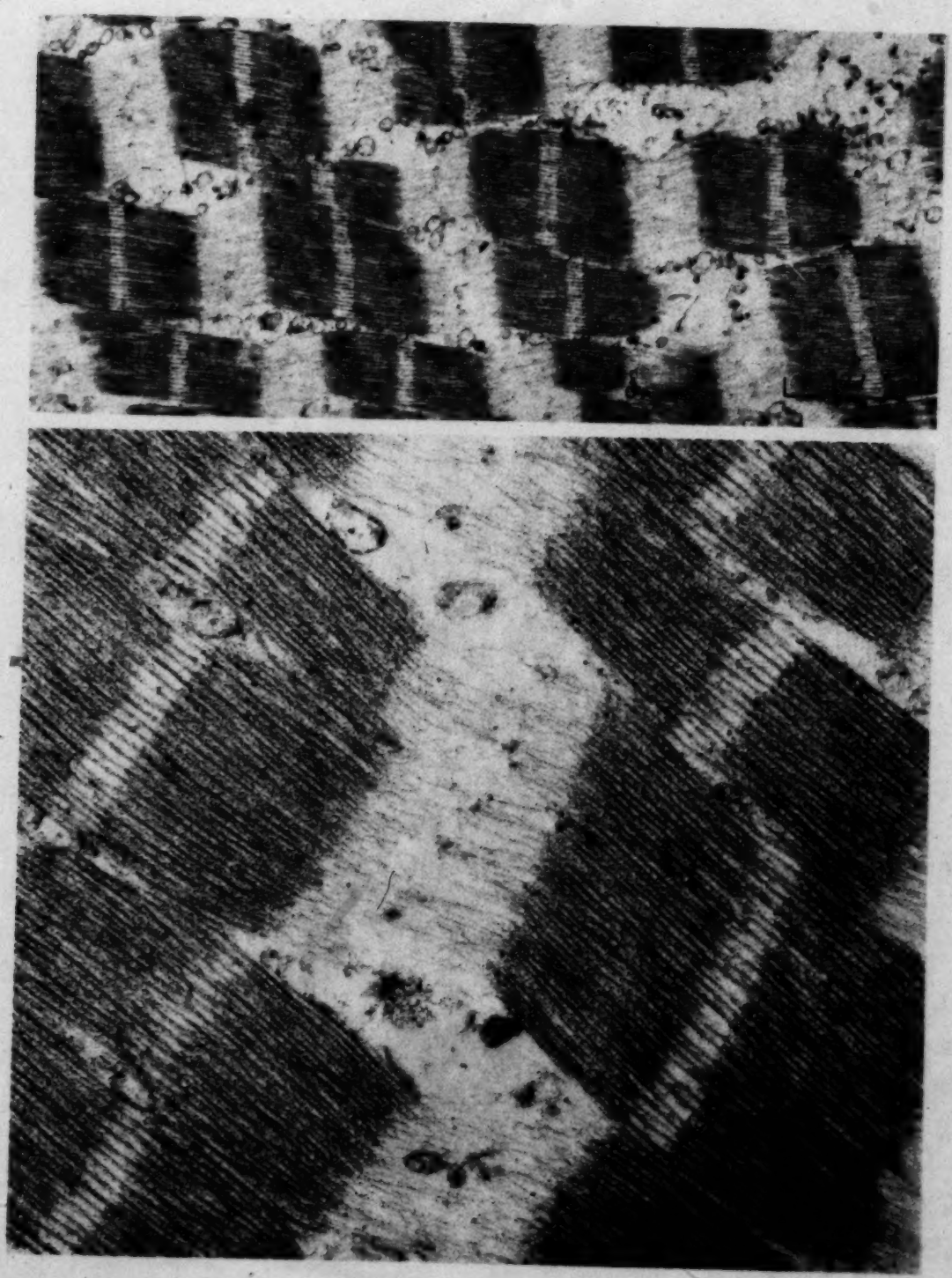

Plate 21. Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM $CaCl_2$.

was disorganized. Since no bands of 41 nm periodicity were observed, hydrolysis of C-protein might have been responsible for the loosening of A-band structure.

The M-line thickening of the thick filaments was reduced and the bare region of the thick filament was exposed. Some residual M-line material remained and cross bridges between the thick filaments were seen in many sarcomeres.

H·S Extracted Fibers

Although H·S is reputed to remove the elements of the thick filament, from these EMs (Plate 22) and gels of similar extractions, it could be seen that much of the thick filament structure remained in the sarcomere; however, most structures of the sarcomere were more disorganized. In the A-band, the A-I junction, and the margins of the pseudo-H-Zone were less distinct, not in register, and of variable width. The M-line appeared as two bands in the pseudo-H-Zone and cross-bridges occurred only at these bands.

The Z-disc structure too had become disorganized, no longer in register, and exhibited gaps in its structure. The precise array of branched filaments was no longer visible. Gels in Plates 4 and 5 had shown that H·S removed the components of both thick and thin filaments. Some solubilization of these filaments may account for the disarray of the A and I bands. Gels have also shown that α-actinin remains unextracted by H·S. These EMs show that Z-disc density remains intact.

In the CAF + Ca^{2+} treated fibers (Plate 23), the Z-disc was absent and the filaments in the I band were again disorganized. No changes were observed in the A-band. Some M-line structure still persisted.

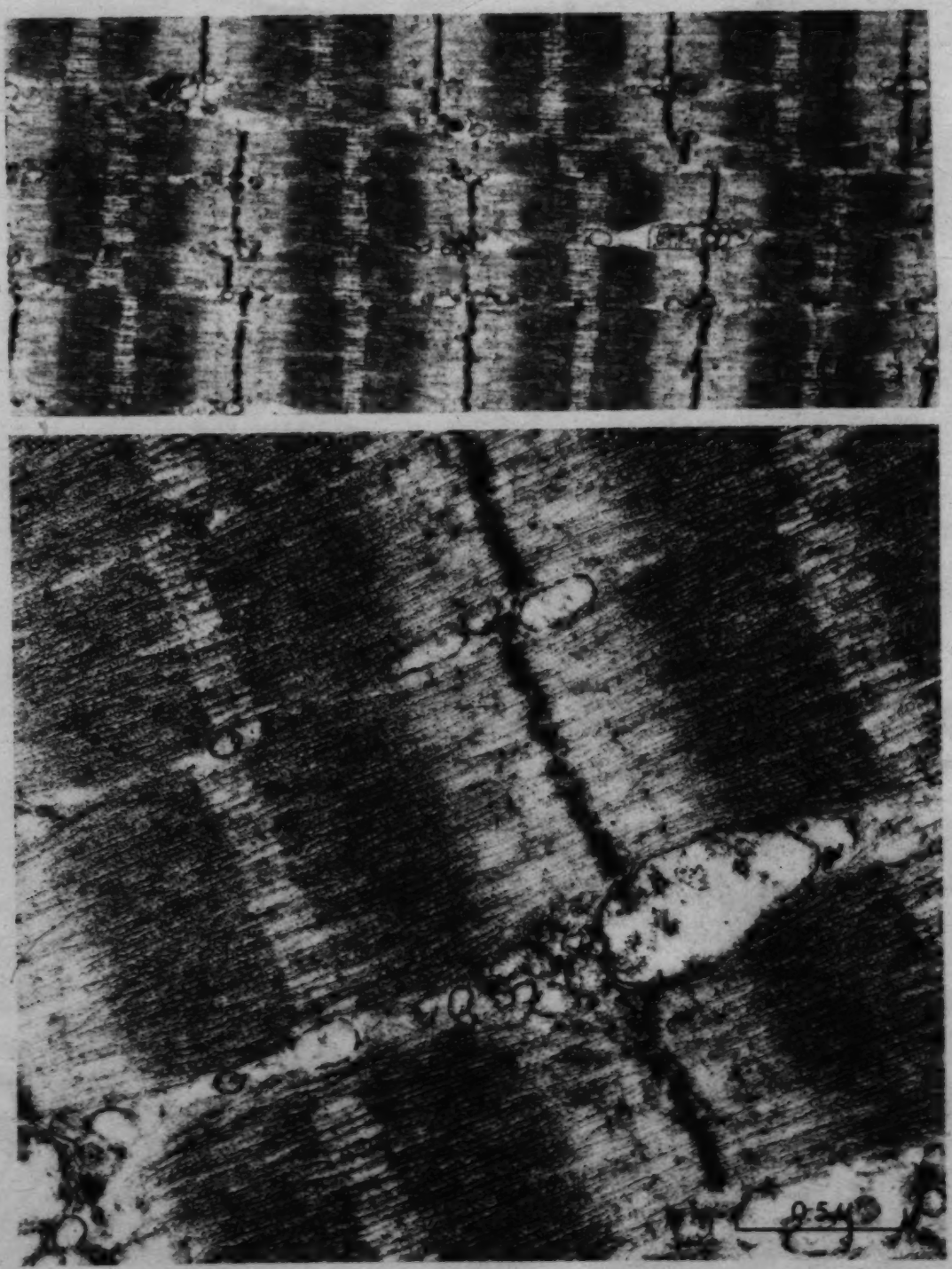

Plate 22. Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM EDTA and subsequently extracted with H-S.

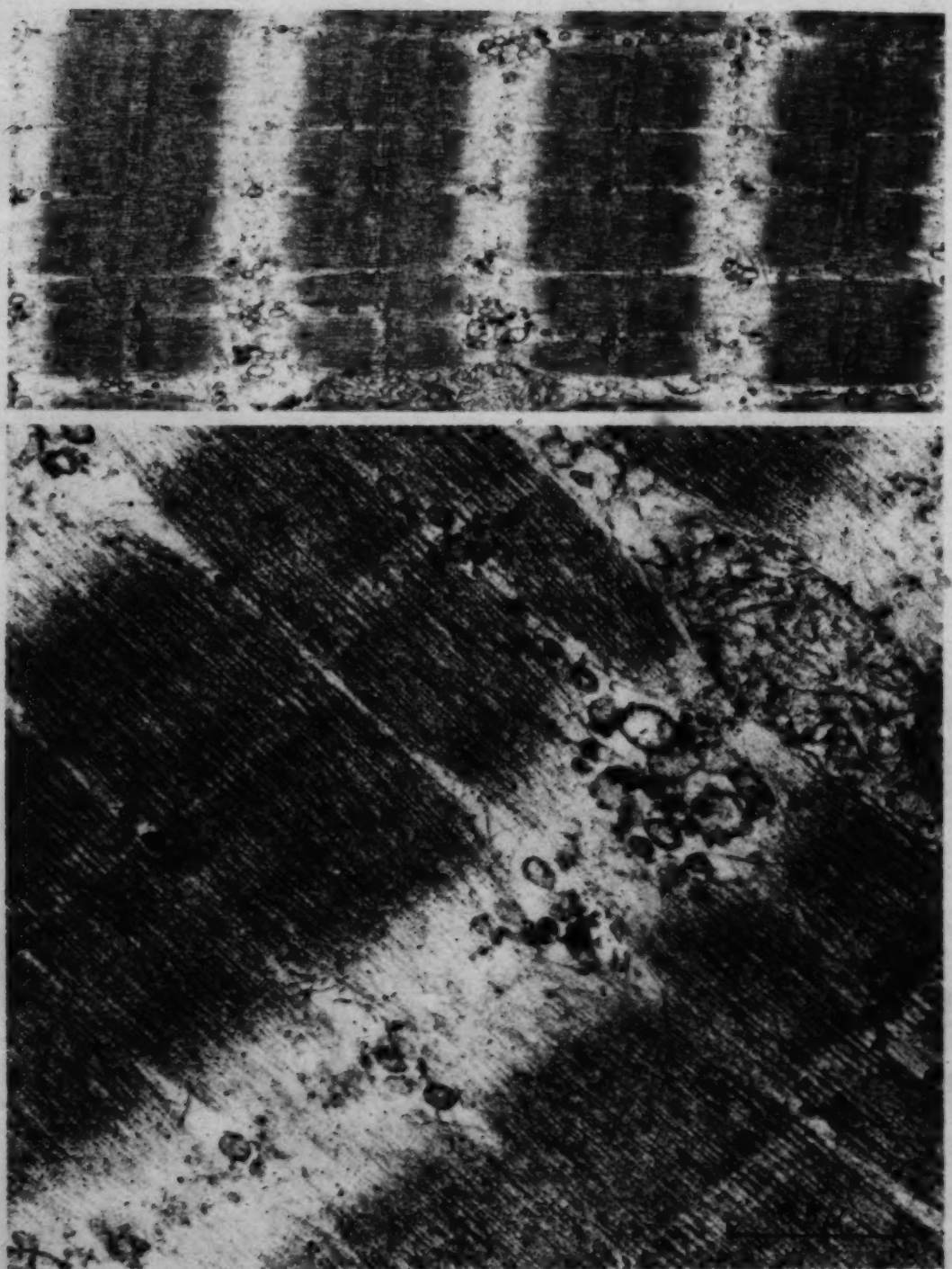

Plate 23. Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM $CaCl_2$ and subsequently extracted with H-S.

KI Extracted Fibers

The entire structure of the myofibril was disrupted in EMs of KI extracted fibers (Plates 24 and 25). No difference was discernible between the control and CAF + Ca^{2+} fibers. Tangled masses of filaments were seen aggregated in two bands at the outer edges of the A-band, only slightly less dense in the centre H-Zone. The I-band identified by the presence of the T-system and SR, also contained tangled thick filaments. No thin filaments were evident. Visible Z-disc structure was completely absent. From the lack of distinct structural features in these fibers, it is difficult to conceptualize the structure of KI extracted fibers from these EMs. These results are in agreement with the results of Locker and Leet (1976) who also observed Z-disc extraction and general disruption of the structure following H·S and KI extraction.

In phase contrast studies presented in this work (Plate 19) and by others (Granger and Lazarides, 1978), the Z-disc structure remained intact and clearly visible. In fact, in fibers homogenized following extraction with KI, the Z-disc structure remains intact and appears to be the sturdiest structure. On the basis of this phase contrast evidence, it is possible therefore that the structure of KI extracted fibers observed in these EMs may have been altered during the preparation of the samples for electronmicroscopy.

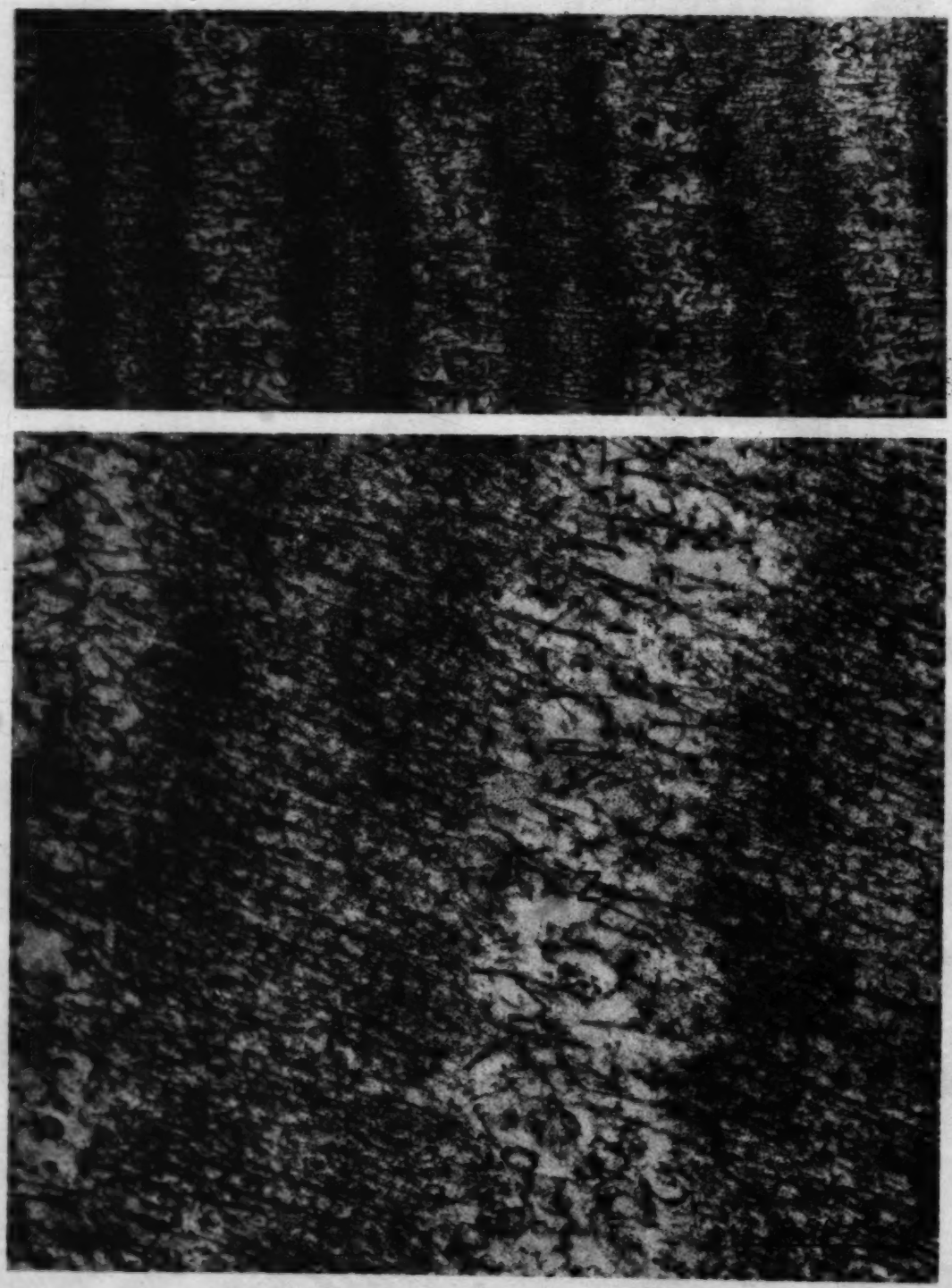

Plate 24. Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM EDTA and subsequently extracted with H-S and KI.

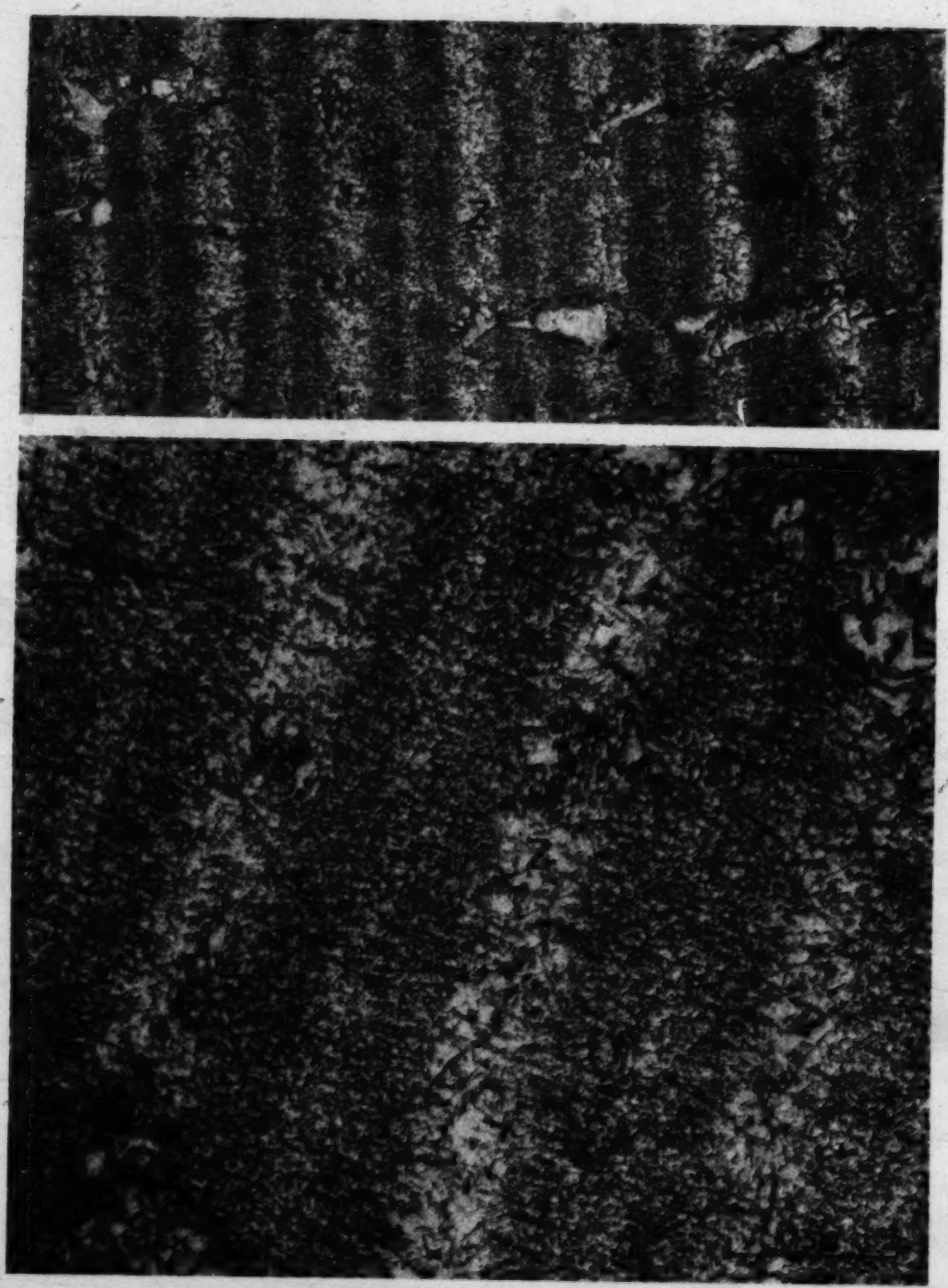

Plate 25. Electron micrographs of glycerinated muscle fibers incubated with CAF and 5 mM $CaCl_2$ and subsequently extracted with H-S and KI.

Chapter 4

DISCUSSION

Since the first reports of Z-disc degradation in post-mortem muscle were published (Davey and Gilbert, 1967; Fukazawa and Yasui, 1967; Stromer, Goll and Roth, 1967), several groups of investigators have studied the phenomenon in an attempt to explain what takes place. In pork, rabbit, and beef muscle (Henderson, Goll and Stromer, 1970), Z-disc degradation was described, but the cause remained uncertain. It was probable the mechanism involved proteolysis, since studies with trypsin also caused similar specific Z-disc removal (Stromer et al., 1967). The observation that complete Z-disc removal occurred in muscle strips incubated in solutions containing 1 mM $CaCl_2$, led to the isolation of the calcium activated proteolytic enzyme with the capability of removing the Z-discs from isolated myofibrils. The isolated enzyme from pork muscle has been shown to cause changes in the structure and proteins of isolated myofibrils similar to those changes observed during aging. The results presented in this thesis verify that changes in myofibril proteins during aging parallel in many aspects the changes produced by CAF activity on isolated myofibrils. Results which led to that conclusion will be discussed in the following section.

Introductory experiments presented here (Plate 3) established that in both CAF treated and aged myofibrils, two reproducible effects were observed in gels of myofibril proteins:

(1) a reduction in the α-actinin band.

(2) the appearance of 30,000 and 27,000 dalton bands.

Of these two major changes, the reduction in the amount of α-actinin remaining in the myofibril is most significant with respect to the investigation of the mechanism of Z-disc degradation, since α-actinin has been shown to exist only in the Z-disc. The results presented here (Plates 4b and 5b) have shown that the α-actinin released has the identical subunit molecular weight (106,000 daltons on this gel system) as in the intact myofibril. These results confirm the extensive work of Dayton et al. (1975) which showed that the molecular weight of α-actinin from pork muscle is not altered on its release from the myofibril. Furthermore, it retains its N-terminal and C-terminal residues and has not been subject to any hydrolysis (Dayton et al., 1975). The underlying postulate of this thesis is that another protein, responsible for the binding of α-actinin to the Z-disc, is hydrolysed or altered in some way by CAF, which causes the release of α-actinin. Several lines of evidence exist to support this idea, drawn from results from this work and from similar experiments published in the literature. These will be presented and discussed in the following section.

Evidence from phase contrast microscope studies on the CAF treatment of myofibrils reported by Dayton et al. (1975) revealed that Z-disc hydrolysis occurs simultaneously as α-actinin solubilization. It was therefore concluded that these events are related. This conclusion is further supported by the fact that the only other detectable proteolysis which occurs is the degradation of TN·T to 30,000 and 27,000 dalton fragments. It seems unlikely that the destruction of the troponin regulatory complex in the thin filament could be responsible for the observed effects

on Z-disc structure.

All three events described above are observed early in the aging of normal post-mortem muscle. Further degradation of additional components occurs if aging or incubation with CAF is prolonged. Hydrolysis of the M-protein (157,000 daltons) was observed in SDS-PAGE patterns after fourteen hours of incubation with CAF (Plate 3). In addition, it is clearly evident in electron micrographs (EMs) in Plate 21 that a loss of M-line structure occurred with CAF incubation. Complete Z-disc removal in the CAF-treated glycerinated fibers was prominent. Dayton et al. have also observed M-protein degradation on gels (1975) and in EMs (1976b), although the phenomenon is not as pronounced in pork muscle as in this work with chicken.

The degradation of TN·I was observed in chicken muscle after the changes in TN·T had become prominent. Ishiura et al. (1979) reported similar results. They also concluded that TN·I was more slowly degraded by CAF than TN·T. They demonstrated this by incubation of isolated chicken troponin with CAF.

In the study of the effects of CAF on myofibril structure, Dayton et al. (1975; 1976b) described the hydrolysis of C-protein as seen in gels of myofibril proteins, and related this to the loss of 43 nm periodicity in the A-bands in EMs of CAF-treated pork muscle fibers. In chicken, little has been published about C-protein. The C-protein from rabbit has a molecular weight of 140,000 (Offer, Moos and Starr, 1973). In chicken leg muscle, a protein of 145,000 daltons has been observed (Gard and Lazarides, 1979) in gels and tentatively identified as C-protein. In the present work, no bands were observed with that molecular weight. Although the C-protein band might be expected to be soluble in H·S

since it is intimately associated with the thick filament, no protein band was observed in such extracts and furthermore, no protein of similar MW was hydrolysed on treatment of the H·S extracted proteins with CAF (Plate 7b). EMs did, however, show a weak periodicity of 41 nm in the A-bands of control fibers. No such detectable periodicity in the CAF + Ca^{2+} treated fibers could be detected. This observation indicates that C-protein hydrolysis cannot be ruled out.

When aging conditions were made more extreme (144 hours at 26°C, Plate 3), drastic changes in the high MW region which suggest myosin degradation were also observed. Ishiura et al. (1979) observed the hydrolysis of purified chicken myosin when subjected to high levels of CAF activity. Under these conditions they also observed the hydrolysis of α-actinin and tropomyosin, but these events were not observed in the results presented here using intact myofibrils. The proteolysis of myosin and the other proteins no doubt occurs when incubation conditions are extreme, but was not observed occurring during normal post-mortem aging at low temperatures.

The preceding results involved the changes in the myofibril and reveal a preferred sequence of proteolysis which reflects the substrate specificity of proteins of the intact myofibril. These events are listed in chronological order:

1. α-actinin is released from the Z-disc matrix; Z-disc structure is observed to dissolve. TN·T is hydrolysed.
2. M-protein and TN·I are hydrolysed. C-protein may also be degraded.
3. Myosin and possibly other proteins are degraded.

The disappearance of observable Z-disc structure and α-actinin release

occur earlier than the other events. It is therefore unlikely that the subsequent proteolysis observed causes Z-disc breakdown.

A compelling explanation of the foregoing observations presumes the existence of a protein in the myofibril, highly susceptible to proteolysis by CAF, which when hydrolysed is responsible for both the release of α-actinin from the Z-disc and the dissolution of observable Z-disc structure. The following discussion presents evidence for both the existence and identity of that myofibril protein.

Myofibrils were extracted using H·S and KI after incubation with CAF to assess the hydrolysis of myofibril protein. The protein composition of extracts of CAF-treated myofibrils differed from controls in several aspects. The changes which pertain to the proteolysis of the Z-disc will be discussed below.

As already described, CAF treatment resulted in the release of α-actinin from the intact myofibril (Plates 4b and 5b). Few differences existed that might account for the changes in the Z-disc structure in gels of the intact myofibril or of H·S extracted proteins of CAF treated and control incubations. Plates 4c, 5c and 6b show that H·S extracts only the thick and thin filament proteins. Myosin, actin, tropomyosin and troponin were all extracted. CAF treated samples differed from controls only in extracted troponins. The 30,000 and 27,000 dalton degradation products of TN·T hydrolysis were soluble in H·S as well as the unhydrolysed troponin complex. Little α-actinin was extracted by H·S, an indication that the Z-disc complex remains insoluble. This fact was clearly demonstrated in EMs of H·S extracted myofibrils (Plate 20) where Z-disc structure remained virtually unaffected.

The H·S insoluble proteins in control incubations consisted

primarily of myosin, α-actinin and actin (Plates 4d and 5d). Tropomyosin and high MW protein was also present in the one hour incubation (Plate 5d). In these CAF treated proteins, a 27,000 dalton band and low molecular weight peptides were observed. It is unlikely that this 27,000 band originated from TN·T hydrolysis, since the intact troponins and their products of proteolysis had been removed in the H·S extraction (as seen in Plates 4c, 5c, 6b). Furthermore, in CAF treated H·S extracted samples, this band has never been observed to be accompanied by the 30,000 dalton component which is always present when TN·T is hydrolysed. In the fourteen hour incubation, less actin and no α-actinin remained in the CAF treated residue. Whether these changes represent actual hydrolysis of the proteins or merely altered solubility in H·S cannot be determined from this experiment. These observations were significant however, since α-actinin and actin are both Z-disc components.

KI extracted about 50% of the remaining proteins (Plates 4e, 5e and 6c). Less actin and α-actinin were present in extracts from CAF treated myofibrils than in similarly incubated controls.

The KI insoluble residues of control myofibrils (Plates 4f, 5f and 6d) contained myosin, α-actinin and actin. In CAF treated or aged myofibrils, α-actinin was completely absent and residual actin was either substantially reduced or in some cases completely removed.

It becomes clear from the examination of the foregoing results that some of the changes cannot be easily explained. Furthermore, not all changes observed (as in the fourteen hour incubation) are Z-disc related. Thus only those changes which may concern the Z-disc components have been discussed.

The nature of the KI-extracted protein complex, was revealed in

studies by Granger and Lazarides (1978). Those results are presented here to introduce the protein composition and structure of the KI insoluble myofibril. Following KI extraction of glycerinated fibers, Granger and Lazarides prepared sheets of Z-discs by homogenization. When viewed end-on, immunofluorescence established that actin and α-actinin existed within the actual Z-disc matrix. Each myofibril was surrounded at the Z-disc by filaments containing actin and desmin. Some additional material was present, seen collapsed onto the Z-disc sheet. This collapsed material contained actin, myosin and tropomyosin and did not appear to exist within the actual Z-disc. This material contained tropomyosin probably because they had not previously extracted their preparations with H·S. From this immunofluorescence evidence it was concluded that some thin filaments protrude from the Z-disc and may be linked to myosin. These observations explain the presence of traces of myosin in gels prepared from ghost protein. The nature of the bond between actin and myosin is unclear; however, Granger and Lazarides suggested that since the extraction was carried out in a non-reduced environment that disulfide bonding may be partially responsible for the interactions of these proteins. Additional evidence of disulfide bridging is evident in the results presented in this thesis in Plate 9, where SDS alone did not solubilize actin and myosin. The subsequent addition of 2-mercaptoethanol did solubilize the residue. Thus, disulfide bridging may exist between actin molecules in the Z-disc. β-mercaptoethanol was not necessary for the solubilization of α-actinin.

In aged samples, it has been suggested that actin may be enzymatically cross linked to myosin (Gard and Lazarides, 1979). An enzymatic cross linking phenomenon between actin and myosin could explain why actin remains in ghosts in aged fibers but is totally extracted in myofibrils incubated with CAF.

The foregoing extraction experiments showed changes in the amount of α-actinin and actin remaining in the KI insoluble myofibrillar material. From these experiments it is difficult to tell whether the two proteins were hydrolysed or simply made more soluble and more easily extracted in H·S or KI. Since little protein actually remains in KI insoluble residues, extraction of additional actin or α-actinin by H·S or KI could easily go undetected.

Subsequent experiments proved, however, that actin in KI insoluble residues was actually hydrolysed by CAF. This was shown repeatedly by incubating proteins from H·S and KI extractions with CAF. The actin, myosin and α-actinin in ghosts were all hydrolysed by CAF (Plate 7e). KI soluble myosin and α-actinin were, however, also hydrolysed. Myosin and α-actinin therefore undergo some alteration (most likely denaturation) upon exposure to KI. As a result, both molecules become completely susceptible to CAF hydrolysis. The KI soluble actin, however, remains refractory to proteolysis in contrast to KI insoluble actin which was readily hydrolysed. KI is known to alter the conformation of many proteins including actin (Szent-Gyorgyi, 1951). However, actin, if completely homogeneous, would be expected to behave consistently. This was not the case. The difference in hydrolysis between the KI soluble and insoluble actin forms showed that unlike myosin and α-actinin, actin behaved as two molecules. The KI soluble fraction was refractory to CAF hydrolysis, and the KI insoluble fraction was easily hydrolysed. Furthermore, gel patterns of ghosts did not change when extraction was extended up to twenty-four hours. The amount of KI insoluble actin that exists in myo-

fibrils is therefore constant and unaffected by KI.

Actin hydrolysis was also indicated in H·S extracted myofibrils where much of the thin filament actin had been solubilized. CAF produced a 27,000 dalton band in H·S extracted myofibrils. A similar 26,000 product resulted from the hydrolysis of purified ghost actin (Plate 13). Thus, the band in H·S extracted myofibrils might originate from the hydrolysis of the KI insoluble form of actin. Similarly, the 27,000 dalton fragment observed in CAF treated myofibrils may, in part, originate from the same hydrolysis.

Thin filament actin, in contrast, is refractory to CAF hydrolysis. Plate 8 clearly showed that CAF hydrolysed neither native G nor F actin. Further, it is obvious in gels of myofibrils after incubation with CAF, that no discernible change in the actin band can be detected. The conclusion can be drawn from these observations that none of the usual forms of actin are hydrolysed by CAF. These results are further verified by similar observations by Dayton et al. (1975) and Ishiura et al. (1979).

In direct contrast, the actin in ghosts was readily hydrolysed. In addition, when the rate of proteolysis of actin in prepared ghosts was compared to the hydrolysis of proteins in intact myofibrils (Plate 13) the rate of disappearance of the actin in ghosts parallelled the appearance of TN·T hydrolysis products, indicating that the ghost actin is a preferred substrate for CAF.

Furthermore, CAF hydrolysed ghost actin with the same efficiency with respect to Ca^{2+} requirement and pH optimum as it hydrolysed other substrates. Studies with ghosts, intact myofibrils, and casein showed all three substrates were hydrolysed with the same efficiency with respect to Ca^{2+} optimum. It was of interest that maximum CAF activity was attained

at lower Ca^{2+} concentrations than for the pork enzyme (1 mM, Dayton et al., 1976b). Furthermore, in parallel incubations of ghosts under identical conditions, CAF hydrolysis of actin in ghosts was detectable to levels as low as 50 μM Ca^{2+} and pH 5.5. Under these conditions, little or no CAF activity was detectable by the measurement of TCA soluble peptides, yet proteolysis of actin by limited hydrolysis was still evident in the gels. Thus, CAF hydrolysis of actin in ghosts occurs at Ca^{2+} levels and at the pH found in post-mortem muscle. CAF hydrolysis of ghost actin under these non-optimum conditions may be a further indication of its substrate specificity.

The possibility existed that the protein in ghosts hydrolysed by CAF might possess the same molecular weight but differ substantially in its composition. For that reason the amino acid composition and isoelectric pH of the ghost protein was compared with that of KI soluble actin to determine whether differences existed.

The results of the amino acid analysis indicated that a great deal of similarity existed in the amino acid composition. The proteins were nearly indistinguishable.

The isoelectric pHs of the two molecules were, however, slightly different. This result reflects a minor difference in net charge between the two molecules, due possibly to undetected minor variations in amino acid composition, or perhaps due to post-translational protein modification. A minor difference in primary structure does not easily explain great differences in the susceptibility of this protein to hydrolysis, especially since CAF has been shown not to prefer any specific sequence of amino acids for its substrate (Ishiura et al., 1979). However, since the primary sequence of a protein dictates its conformation, it is pos-

sible that the actin in ghosts exists in an entirely different form than thin filament actin. A well-known example of minor heterogeneity in proteins has been illustrated in the analysis of sickle-cell anemia (Ingram, 1957). Hemoglobin S from patients who possess the sickle-cell trait, was found to differ in isoelectric pH, and differed from the normal hemoglobin A by a single amino acid substitution. The alteration markedly changes the properties of hemoglobin S.

In myofibrils it has been shown that the conformation of the substrate is essential to its susceptibility to hydrolysis by CAF. Actin, myosin, α-actinin, and to a lesser extent tropomyosin, all resist hydrolysis in their native state. However, once denatured, they are all easily hydrolysed by CAF (Plate 14). These results agree with Ishiura et al. (1979) who concluded that "CAF digests most proteins once they are denatured."

The importance of three-dimensional protein structure of the substrate is emphasized here by the fact that all of these molecules possess CAF-susceptible sites but they are not hydrolysed when in their native form. By simply denaturing the protein structure nearly all proteins become hydrolysable.

These results imply that the susceptibility to CAF hydrolysis observed in ghost actin might be due to its different conformation in the Z-disc, assembly and only indirectly due to the difference in primary structure as detected by IEF. This concept is supported by the fact that ghost actin and KI soluble actin, following isolation by preparative SDS-PAGE (Plate 13), were both hydrolysed virtually identically. This result shows that ghost actin does not possess some CAF susceptible site that is not present in normal thin filament actin.

On the basis of the similarity in amino acid composition, iso-

electric point and molecular weight, it is likely that KI extracted myofibrils contain a variant of thin filament actin that exists in the Z-discs of myofibrils. Its conformation differs from that normally found in thin filament actin in that the molecule exists in the correct conformation to permit hydrolysis by CAF.

The results presented here in detail for chicken have been in part confirmed using beef myofibrils and beef CAF. Low and unstable CAF activity precluded extensive studies of the beef system. However, the results of the preliminary beef work confirmed that CAF was behaving in a similar fashion. It is possible that ghost actin is a universal component of striated muscle Z-discs.

Lazarides and Hubbard (1976) also have identified a protein from chicken muscle insoluble in KI, judged to be actin on the basis of its molecular weight, its reaction with antibodies against smooth muscle actin, and by its isoelectric point.

Purified KI-soluble and ghost actins were used to immunize mice in order to elicit antibody production. Mice failed to respond to the KI soluble antigen. Antisera to ghost actin was produced however, and reacted identically to both proteins. Possible explanations for the observed results were discussed in Chapter 3. Since care was taken to isolate both proteins identically using preparative SDS gel electrophoresis, the denaturing due to SDS should also be the same. Yet KI soluble actin did not elicit an antigenic response suggesting that differences in actin primary structure were responsible for the recognition of Z-disc actin as a foreign protein. Further studies will be necessary to prove this point.

The antiserum developed against purified ghost actin was used

in indirect immunofluorescence experiments to locate Z-disc actin and demonstrate its hydrolysis. These results clearly illustrate the essential conclusions of this work and can be effectively used as a summary.

Immunofluorescence results definitively show differences in the location of two varieties of actin within the myofibril structure. Fluorescent actin labelling in intact myofibrils existed across the entire sarcomere. Particulary enhanced fluorescence was observed at the Z-disc, and might be explained by a greater concentration of actin at the Z-disc or by a greater affinity of the antibody preparation for actin in the Z-disc. The latter possibility cannot be ruled out, because the differences in actin structure reflected by IEF could result in the exposure of different antibody recognition sites. Following KI extraction, Z-disc fluorescence remained unaffected but fluorescence between Z-discs was removed. This result established that KI insoluble actin is located in the Z-disc and is unaffected by KI extraction. Conversely, thin filament actin is KI soluble.

In CAF treated myofibrils, the fluorescence at the Z-disc was completely removed. This single result clearly establishes that Z-disc actin is hydrolysed in intact myofibrils. Since thin filament fluorescence was unaffected, KI soluble thin filament actin is therefore refractory to CAF hydrolysis.

The conclusion that can be drawn from these experiments is that at least two different forms of actin exist within the myofibril. One form exists in the thin filament, and comprises most of the myofibrillar actin. A small amount of actin exists in the Z-disc and differs from the thin filament form in that it is susceptible to hydrolysis by CAF. It also differs in solubility in KI, isoelectric pH, and antigenicity

in mice.

A considerable body of published evidence now confirms that a variety of forms of actin exist in muscle distinguishable by differences in isoelectric pH. In striated muscle, three primary forms, α, β, and γ actin were identified by two-dimensional electrophoresis (Izant and Lazarides, 1977; Rubenstein and Spudich, 1977; Whalen, Butler-Browne and Gros, 1976). These three forms were further evaluated by sequencing (Vandekerckhove and Weber, 1978). β and γ-actin were found to differ from the α form by twenty-five and twenty-four residues respectively. Most substitutions involved uncharged amino acids. The predominant form, α-actin, exists in the thin filament, and is responsible for contraction. α-actin is soluble in KI. Izant and Lazarides (1977) found that the residue of KI extracted myofibrils contained primarily γ-actin. They also observed its unusual insolubility, but were unable to explain it.

In smooth muscle as well, γ-actin exists but in a greater proportion than in striated muscle. The results presented in Plate 16 have shown that actin from smooth muscle is partly CAF-hydrolysable, and that all the KI insoluble actin was hydrolysed. Therefore in smooth as well as in striated muscle, there exists a proportion of the actin that is susceptible to CAF hydrolysis. The CAF-hydrolysable actin might therefore be the γ-form.

The functions of the three identified isoelectric variants have yet to be established, but the results presented here concur with Izant and Lazarides (1977) who, based on information gathered on the KI insolubility and the association of γ-actin with structural proteins, concluded that γ-actin may have a structural function.

In later studies, Granger and Lazarides (1978) showed that actin resides in Z-discs of KI extracted fibers, using anti-thin-filament actin antibodies.

Several additional published results bear out that a different form of actin exists in the Z-disc. Gard and Lazarides (1979) used guinea pig transglutaminase to attach fluorescent labels to all myofibril Z-disc proteins. Their results showed that a substantial proportion of the label was incorporated into actin in the Z-disc. Light trypsin digestion of myofibrils eliminated Z-disc fluorescence. In the subsequent analysis of myofibril proteins, the fluorescent-labelled actin had been hydrolysed, and α-actinin had been released unhydrolysed into the supernatant solution. Trypsin has been used in the study of Z-disc degradation (Stromer et al., 1967) because its effects on Z-disc structure are similar to those of aging. These studies also verify that the actin in Z-discs is extremely susceptible to proteolysis and that its hydrolysis is directly linked to the release of α-actinin.

The current models of the vertebrate Z-disc, based on ultrastructural studies (Ullrick et al., 1977), propose that the thin filament loops into the Z-disc. In the Z-disc, the thin filament associated with an "amorphous mass" thought to consist of α-actinin.

Binding studies at physiological temperatures (37^{o}C) have shown that α-actinin binds one or more thin filaments at only one end of the filament in the presence of tropomyosin (Goll, Suzuki, Temple and Holmes, 1972). Moreover, since Huxley (1963) had shown that the F-actin strand was polarized, Goll's model therefore supposed that α-actinin bound intact myofibrils only at the Z-disc in the presence of tropomyosin. Some difference existed therefore at the Z-disc terminus of the thick

filament which prevented tropomyosin binding.

Stromer et al. (1972) speculated that the native thin filament might possess a unique conformation or different proteins near the Z-disc terminus. In earlier work, Goll et al. (1969) noted an area of greater density and straightness in thin filaments adjacent to the Z-disc.

The results of this study indicate that an actin molecule of different conformation may exist at the Z-disc terminus of the thin filament, that by virtue of its different structure may be responsible for the observed differences in thin filament shape and affinities for both tropomyosin and α-actinin, and hence contribute to the structural stability of the Z-disc.

Very recently, Kuroda, Tanaka and Masaki (1981) published a report of the isolation of a protein which they called eu-actinin, extracted from myosin-poor H·S extracted myofibrils, using a low ionic strength (5 mM Tris-maleate pH 6.5) buffer. Their protein has some similar characteristics to the Z-disc actin prepared in this work. "Eu-actinin" has an identical molecular weight to actin but varies in amino acid composition and isoelectric pH. Antibodies prepared against eu-actinin, however, did not react with native actin or two kinds of denatured actin. SDS-denatured actin was not used.

Direct immunofluorescence using antibodies to eu-actinin revealed fluorescence at the Z-disc in chicken myofibrils which was not removed by KI extraction but was removed by incubation with CAF from chicken cardiac muscle. They further showed using affinity chromatography that eu-actinin binds α-actinin strongly, and F-actin with less affinity. Eu-actinin did not bind tropomyosin. On the basis of these results, it is possible that this isolated protein is the same as the actin isolated in

this work.

SUMMARY

Results have been presented that identify a KI insoluble form of actin which resides in the Z-disc of striated muscle and is susceptible to proteolysis by CAF. The Z-disc actin differed from the usual thin filament actin in solubility in KI, isoelectric pH and antigenicity in mice. It was concluded that differences in conformation may give rise to its unusual properties.

The hydrolysis of Z-disc actin was linked with degeneration of Z-disc and is likely the cause of the dissolution of observable Z-disc structure and the release of α-actinin during post-mortem aging in muscle. Z-disc actin therefore contributes to the strength and stability of the Z-disc and is essential in the integrity of the entire myofibril structure.

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APPENDICES

APPENDIX A

Purification of CAF from Chicken Breast Muscle

Purification of CAF from Chicken Breast Muscle

The enzyme CAF was purified by slight modification of the procedure outlined in a short communication by Ishiura et al. (1978). The following is a complete description of the procedure used to isolate CAF to electrophoretic purity following that method.

Minced breast muscle was prepared as previously described in materials and methods. Five hundred to 1000 g minced muscle were homogenized for thirty seconds with three volumes of 20 mM $NaHCO_3$ containing 1 mM EDTA in a cold Waring blendor. The suspension was then centrifuged at 15,000 xg for fifteen minutes. The pellets were discarded. The pH of the clear red supernatant fluid was slowly adjusted from near pH 6.4 to pH 4.9 using acetic acid (diluted 1:3 with H_20) with stirring on ice. The proteins were allowed to precipitate by standing at 0^oC for fifteen minutes. The suspension was again centrifuged for fifteen minutes at 6500 xg for thirty minutes. The collected pellet was resuspended in about 80 ml of 20 mM tris-HCl, 0.1 M NaCl, 5 mM EDTA and 10 mM 2-mercaptoethanol pH 7.0 (Buffer A), using a teflon ball homogenizer. The pH of the suspension was adjusted to 7.0 and the mixture was stirred for two hours to allow the protein to go back into solution. Insoluble material was removed by centrifugation at 31,000 xg in a JA-20 rotor. The supernatant fluid was then applied to a DEAE-cellulose (DE-23, Whatman Ltd.) column and eluted with a 0.1 M to 0.6 M NaCl gradient (Figure 9). One major peak of enzyme activity eluted with a NaCl concentration of 0.34 M. NaCl concentration measured by conductivity (conductivity meter CDM2d, Radiometer, Copenhagen) using a NaCl standard curve in the appropriate buffer. The column fractions were assayed as described in Materials and Methods.

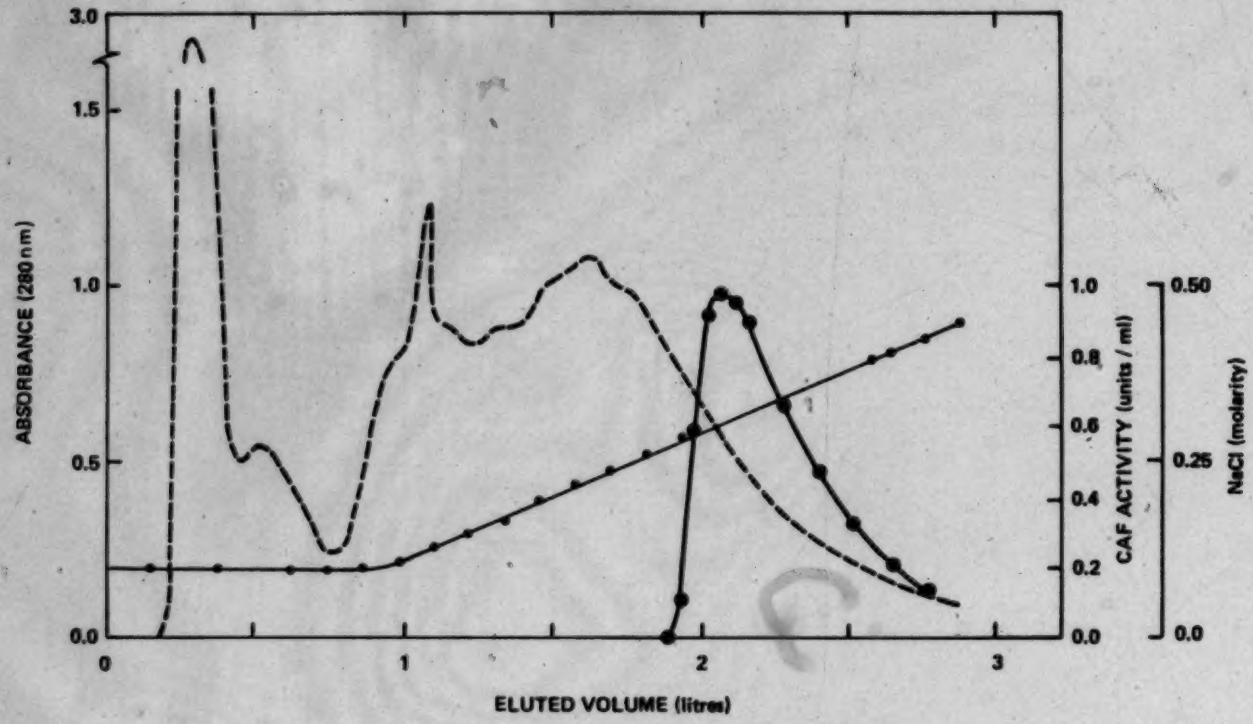

Figure 9. Chromatography of the crude ammonium sulfate fraction containing CAF activity on DEAE-cellulose. 75 ml of ammonium sulfate fraction containing approximately 2000 mg protein in buffer A was applied to a DEAE column (4.5 x 23.4 cm) preequilibrated in the same buffer. The protein was washed on with an additional 100 ml and the column was then eluted with a linear 0.1 to 0.6 M NaCl gradient all in the same buffer in a total volume of 2 l. 17.5 ml fractions were collected. The flow rate was 108 ml/hour. CAF activity was eluted with an NaCl molarity of 0.32 M (determined by conductivity). Fractions containing greater than 0.2 units/ml were combined and concentrated.

(– – – – – –), absorbance at 280 nm.
(●——●), CAF activity.
(●——●), NaCl concentration.

Column fractions containing greater than 0.2 units activity/ml were combined and concentrated by precipitating the protein by adding ammonium sulfate to 60% saturation. After fifteen minutes, the precipitate was centrifuged at 30,000 xg in a JA-14 rotor. The pellet was resuspended by adding 4 ml of 0.1 M NaCl, .02 M tris-HCl, 5 mM EDTA and 10 mM 2-mercaptoethanol pH 7.5 (Buffer B) and dialysed against the same solution overnight. The solution was then clarified by centrifugation at 17,000 xg for ten minutes, then concentrated to a volume of 2.0 ml using a Minicon B-15 concentrator (Amicon Corp., Lexington, Mass.). The concentrated solution was applied to a Sepharose 6B (Pharmacia, Uppsala, Sweden) column and eluted with Buffer B (Figure 10). The eluted fractions were again assayed and those fractions containing activity greater than 0.2 units/ml were applied directly to a small DEAE cellulose column equilibrated in Buffer B. The column was again eluted with a linear NaCl gradient (Figure 11). Active fractions were pooled and concentrated using a Millipore Immersible CX (Millipore Corp., Bedford, Mass.) to a volume near 10 ml. The solution was then diluted 1:1 (v/v) with glycerol and stored in liquid N_2. The enzyme could be stored in N_2 indefinitely without detectable loss in activity. However, when stored at $-15^{o}C$ preparations gradually lost activity and could only be preserved for one to two months.

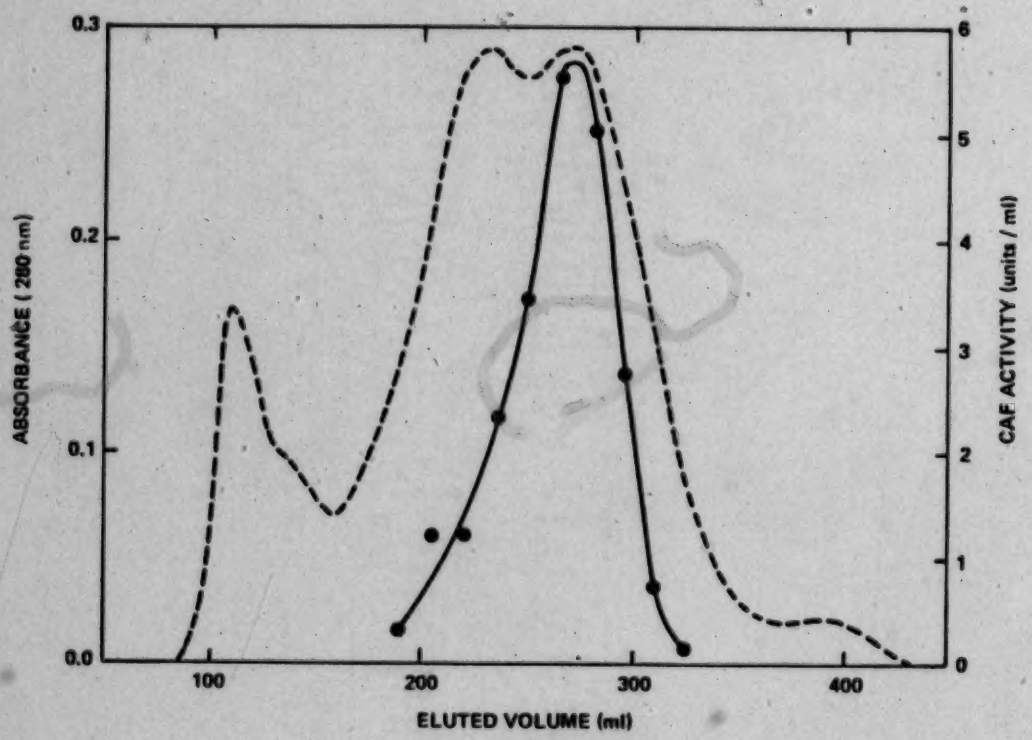

Figure 10. Elution profile of DEAE-purified CAF on Sepharose 6B. The concentrated protein (120 mg in 2.5 ml) containing CAF activity from the first DEAE column was applied to a 2.2 x 60.2 cm DEAE column preequilibrated in buffer A. The protein was eluted at a flow rate of 30 ml/hr collecting 2.5 ml fractions. Those fractions containing CAF activity of greater than 0.1 units/ml were combined.

(------), absorbance at 280 nm.
(●——●), CAF activity.

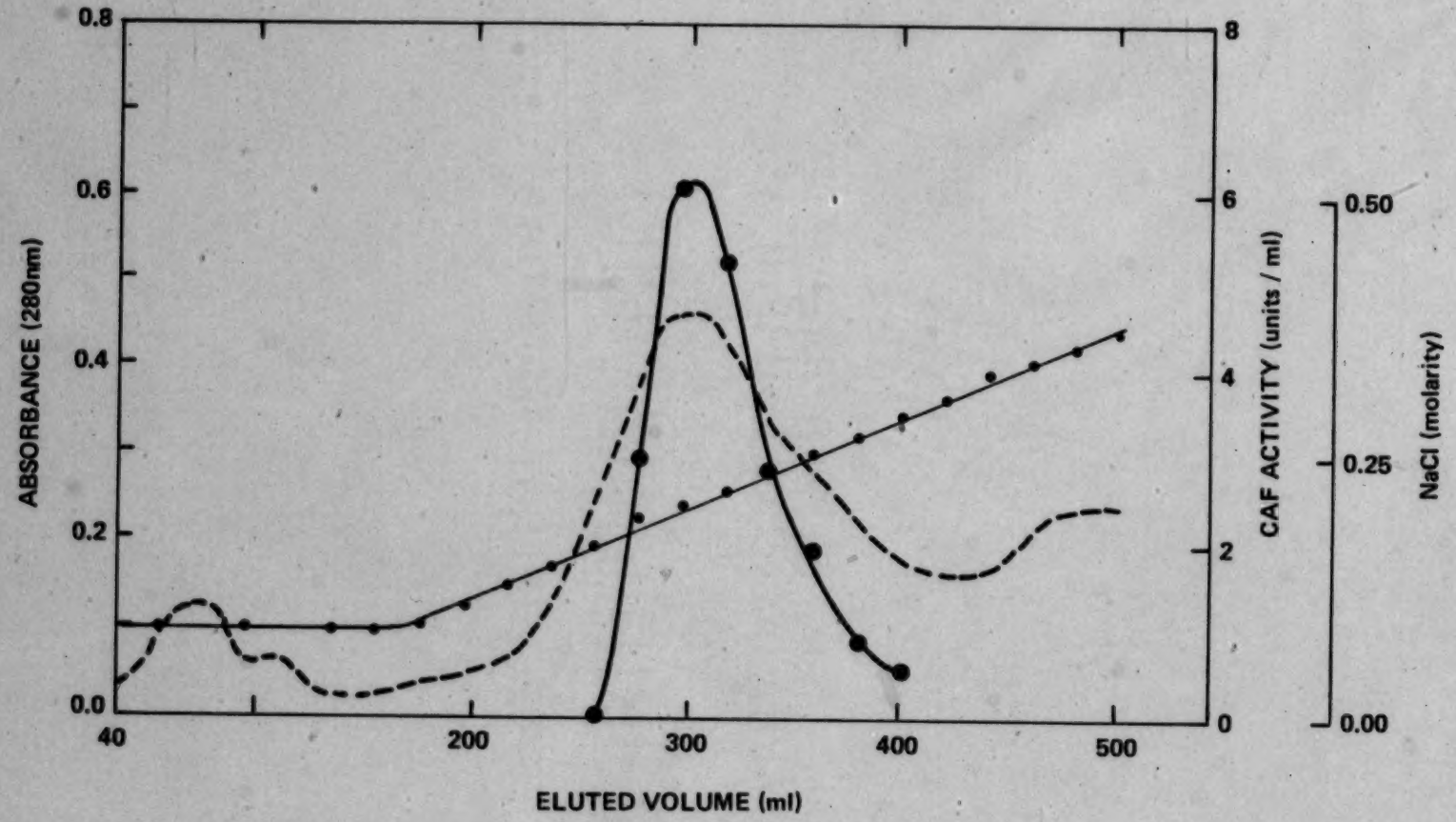

Figure 11. Elution profile of chromatography of Sepharose purified CAF on a second DEAE column. The total sample eluted from the previous Sepharose 6B column (27 ml containing 3.7 mg protein) was loaded on a small (1.3 x 20 cm) DEAE column preequilibrated in buffer B. The protein was washed on with an additional 70 ml then eluted with a 0.1 to 0.6 M NaCl gradient in the same buffer in a total of 500 ml. Column flow rate was 48 ml/hour. 2 ml fractions were collected.

(------), absorbance at 280 nm.

(●——●), CAF activity.

(●——●), NaCl concentration.

APPENDIX B

Extraction of CAF from Beef Muscle

Extraction of CAF from Beef Muscle

Longissimus dorsi or semitendinosus from freshly slaughtered steers were obtained from a local slaughterhouse (Gainers Meats, Edmonton, Alberta) about thirty minutes post-mortem. Muscle was finely minced using a Moulinex food processor then suspended in five volumes of 4 mM EDTA using a Waring blendor. The suspension was centrifuged at 9800 xg for forty minutes. The supernatant fluid was decanted and filtered through glass wool previously rinsed with distilled H_2O. The pH of the clear pink solution was slowly adjusted from near pH 6.1 to pH 4.9 using acetic acid (diluted 1:4). Proteins were allowed to precipitate for ten minutes at $0^{\circ}C$., then the suspension was again centrifuged at 6500 xg for twenty minutes. The protein pellet was then resuspended in a small volume of 0.1 M tris-acetate, 5 mM EDTA and 5 mM dithiothreitol (DTT) pH 8.2 and the pH of the suspension was adjusted to 7.5. The protein suspension was stirred on ice for three hours, clarified for two hours at 140,000 xg using a Beckman type 40 rotor, then dialysed against 1 mM $KHCO_3$, 5 mM EDTA, 5 mM DTT pH 7.7. This solution containing the CAF activity was then mixed with glycerol (1:1) and stored at $-15^{\circ}C$ until use.

APPENDIX C

Preequilibration of Dowex AG1-X2 Acetate Ion Exchange Resin

Preequilibration of Dowex AG1-X2 Acetate Ion Exchange Resin

Dowex AG1-X2 (200-400 mesh) was converted to the acetate form using the following procedure. The resin was suspended in an equal volume of 0.5M NaOH for fifteen minutes then filtered using a sintered glass funnel. The resin was then likewise resuspended in 0.5M acetic acid for fifteen minutes. The resin was then filtered on a sintered glass funnel and washed by resuspension with distilled water until the effluent was neutral. The resin was then resuspended in 0.05M tris-acetate buffer, pH 7.8 and refrigerated in that buffer at 2^{o} until use.